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INDIVIDUALIZATION OF THERAPY WITH ANTICOAGULANTS

RELATED APPLICATION

This application is a new application which claims the benefit of U.S. Provisional Application No. 60/391,976, filed on June 28, 2002. The entire teachings of the above application are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The invention relates to a system and method for individualization of therapy with anticoagulants. More specifically, the present invention relates to the use of metabolic phenotyping in individualizing treatment with anticoagulants.

For the majority of drugs (or xenobiotics) administered to humans, their fate is to be metabolized in the liver, into a form less toxic and lipophilic with their subsequent excretion in the urine. Their metabolism involves two systems (Phase I and Phase II) which act consecutively: Phase I enzymes include the cytochrome P450 system which includes at least 20 enzymes catalyzing oxidation reactions as well as carboxylesterase, amindases, epoxide hydrolase, quinine reductase, alcohol and aldehyde dehydrogenase, xanthine oxidase and flavin-

containing monooxygenase. These enzymes are localized in the microsomal fraction. Phase II enzymes include the conjugation system which involves at least 5 enzymes including, N-acetyltransferases (NAT), UDP-glucoronyltransferases (UGT), sulfotransferases (SUT), and glutathione-S-transferases (GST). A detailed description of the complex human drug metabolizing systems is provided in Kumar and Surapaneni (Medicinal Res. Rev. (2001) 21(5):397-411) and patent application WO 01/59127 A2.

The metabolism of a drug and its movement through the body (pharmacokinetics) are important in determining its effects, toxicity, and interactions with other drugs. The three processes governing pharmacokinetics are the absorption of the drug, distribution to various tissues, and elimination of drug metabolites. These processes are intimately coupled to drug metabolism, since a variety of metabolic modifications alter most of the physicochemical and pharmacological properties of drugs, including solubility, binding to receptors, and excretion rates. The metabolic pathways which modify drugs also accept a variety of naturally occurring substrates such as steroids, fatty acids, prostaglandins, leukotrienes, and vitamins. The enzymes in these pathways are therefore important sites of biochemical and pharmacological interaction between natural compounds, drugs, carcinogens, mutagens, and xenobiotics.

It has long been appreciated that inherited differences in drug metabolism lead to drastically different levels of drug efficacy and toxicity among

individuals. For drugs with narrow therapeutic indices, or drugs which require bioactivation (such as codeine), these polymorphisms can be critical. Moreover, promising new drugs are frequently eliminated in clinical trials based on toxicities which may only affect a segment of the individuals in a target group. Advances in pharmacogenomics research, of which drug metabolizing enzymes constitute an important part, are promising to expand the tools and information that can be brought to bear on questions of drug efficacy and toxicity (See Evans, W. E. and R. V. Relling (1999) Science 286: 487-491).

Drug metabolic reactions are categorized as Phase I, which functionalize the drug molecule and prepare it for further metabolism, and Phase II, which are conjugative. In general, Phase I reaction products are partially or fully inactive, and Phase II reaction products are the chief excreted species. However, Phase I reaction products are sometimes more active than the original administered drugs; this metabolic activation principle is exploited by pro-drugs (e. g. L-dopa). Additionally, some nontoxic compounds (e. g. atlatoxin, benzo [a] pyrene) are metabolized to toxic intermediates through these pathways. Phase I reactions are usually rate-limiting in drug metabolism. Prior exposure to the compound, or other compounds, can induce the expression of Phase I enzymes however, and thereby increase substrate flux through the metabolic pathways. (See Klassen, C. D.,Amdur, M. O. and J. Doull (1996) Casarett and Doull's Toxicology : The Basic Science of Poisons, McGraw-Hill,

New York, NY, pp. 113-186; Katzung, B. G. (1995) Basic and Clinical Pharmacology, Appleton and Lange, Norwalk, CT, pp. 48-59; Gibson, G. G. and Skett, P. (1994) Introduction to Drug Metabolism, Blackie Academic and Professional, London.)

Drug metabolizing enzymes (DMEs) have broad substrate specificities. This can be contrasted to the immune system, where a large and diverse population of antibodies is highly specific for their antigens. The ability of DMEs to metabolize a wide variety of molecules creates the potential for drug interactions at the level of metabolism. For example, the induction of a DME by one compound may affect the metabolism of another compound by the enzyme.

DMEs have been classified according to the type of reaction they catalyze and the cofactors involved. The major classes of Phase I enzymes include, but are not limited to, cytochrome P450 and flavin-containing monooxygenase. Other enzyme classes involved in Phase 1-type catalytic cycles and reactions include, but are not limited to, NADPH cytochrome P450 reductase (CPR), the microsomal cytochrome b5/NADH cytochrome b5 reductase system, the ferredoxin/ferredoxin reductase redox pair, aldo/keto reductases, and alcohol dehydrogenases. The major classes of Phase II enzymes include, but are not limited to, UDP glucuronyltransferase, sulfotransferase, glutathione S-transferase, N-acyltransferase, and N-acetyl transferase.

CYTOCHROME P450 AND P450 CATALYTIC CYCLE-ASSOCIATED ENZYMES

Members of the cytochrome P450 superfamily of enzymes catalyze the oxidative metabolism of a variety of substrates, including natural compounds such as steroids, fatty acids, prostaglandins, leukotrienes, and vitamins, as well as drugs, carcinogens, mutagens, and xenobiotics. Cytochromes P450, also known as P450 heme-thiolate proteins, usually act as terminal oxidases in multi-component electron transfer chains, called P450-containing monooxygenase systems. Specific reactions catalyzed include hydroxylation, epoxidation, N-oxidation, sulfooxidation, N-, S-, and dealkylations, desulfation, deamination, and reduction of azo, nitro, and N-oxide groups. These reactions are involved in steroidogenesis of glucocorticoids, cortisols, estrogens, and androgens in animals; insecticide resistance in insects; herbicide resistance and flower coloring in plants; and environmental bioremediation by microorganisms. Cytochrome P450 actions on drugs, carcinogens, mutagens, and xenobiotics can result in detoxification or in conversion of the substance to a more toxic product. Cytochromes P450 are abundant in the liver, but also occur in other tissues; the enzymes are located in microsomes. (Graham-Lorence, S. and Peterson, J. A. (1996) FASEB J. 10: 206-214.)

Four hundred cytochromes P450 have been identified in diverse organisms including bacteria, fungi, plants, and animals (Graham-Lorence, supra). The B-class is found

in prokaryotes and fungi, while the E-class is found in bacteria, plants, insects, vertebrates, and mammals. Five subclasses or groups are found within the larger family of E-class cytochromes P450.

All cytochromes P450 use a heme cofactor and share structural attributes. Most cytochromes P450 are 400 to 530 amino acids in length. The secondary structure of the enzyme is about 70% alpha-helical and about 22% beta-sheet. The region around the heme-binding site in the C-terminal part of the protein is conserved among cytochromes P450. A ten amino acid signature sequence in this heme-iron ligand region has been identified which includes a conserved cysteine involved in binding the heme iron in the fifth coordination site. In eukaryotic cytochromes P450, a membrane-spanning region is usually found in the first 15-20 amino acids of the protein, generally consisting of approximately 15 hydrophobic residues followed by a positively charged residue (Graham-Lorence, supra.).

Cytochrome P450 enzymes are involved in cell proliferation and development. The enzymes have roles in chemical mutagenesis and carcinogenesis by metabolizing chemicals to reactive intermediates that form adducts with DNA (Nebert, D. W. and Gonzalez, F. J. (1987) Ann. Rev. Biochem. 56: 945-993). These adducts can cause nucleotide changes and DNA rearrangements that lead to oncogenesis. Cytochrome P450 expression in liver and other tissues is induced by xenobiotics such as polycyclic aromatic hydrocarbons, peroxisomal proliferators, phenobarbital,

and the glucocorticoid dexamethasone (Dogra, S. C. *et al.* (1998) Clin. Exp. Pharmacol. Physiol. 25: 1-9). A cytochrome P450 protein may participate in eye development as mutations in the P450 gene CYP1B1 cause primary congenital glaucoma.

Cytochromes P450 are associated with inflammation and infection. Hepatic cytochrome P450 activities are profoundly affected by various infections and inflammatory stimuli, some of which are suppressed and some induced (Morgan, E. T. (1997) Drug Metab. Rev. 29: 1129-1188). Effects observed *in vivo* can be mimicked by proinflammatory cytokines and interferons. Autoantibodies to two cytochrome P450 proteins were found in individuals with autoimmune polyenodocrinopathy-candidiasis-ectodermal dystrophy (APECED), a polyglandular autoimmune syndrome.

Mutations in cytochromes P450 have been linked to metabolic disorders, including congenital adrenal hyperplasia, the most common adrenal disorder of infancy and childhood; pseudovitamin deficiency rickets; cerebrotendinous xanthomatosis, a lipid storage disease characterized by progressive neurologic dysfunction, premature atherosclerosis, and cataracts; and an inherited resistance to the anticoagulant drugs coumarin and warfarin (Isselbacher, K. J. *et al.* (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, Inc. New York, NY, pp. 1968-1970; Takeyama, K. *et al.* (1997) Science 277: 1827-1830; Kitanaka, S. *et al.* (1998) N. Engl. J. Med. 338: 653-661). Extremely high levels of expression of the cytochrome P450 protein aromatase were

found in a fibrolamellar hepatocellular carcinoma from a boy with severe gynecomastia (feminization) (Agarwal, V. R. (1998) J. Clin. Endocrinol. Metab. 83: 1797-1800).

The cytochrome P450 catalytic cycle is completed through reduction of cytochrome P450 by NADPH cytochrome P450 reductase (CPR). Another microsomal electron transport system consisting of cytochrome b5 and NADPH cytochrome b5 reductase has been widely viewed as a minor contributor of electrons to the cytochrome P450 catalytic cycle. However, a recent report by Lamb, D. C. *et al.* (1999 FEBS Lett. 462: 283-8) identifies a *Candida albicans* cytochrome P450 (CYP51) which can be efficiently reduced and supported by the microsomal cytochrome b5/NADPH cytochrome b5 reductase system. Therefore, there are likely many cytochromes P450 which are supported by this alternative electron donor system.

Cytochrome b5 reductase is also responsible for the reduction of oxidized hemoglobin (methemoglobin, or ferrihemoglobin, which is unable to carry oxygen) to the active hemoglobin (ferrohemoglobin) in red blood cells. Methemoglobinemia results when there is a high level of oxidant drugs or an abnormal hemoglobin (hemoglobin M) which is not efficiently reduced. Methemoglobinemia can also result from a hereditary deficiency in red cell cytochrome b5 reductase (Reviewed in Mansour, A. and Lurie, A. A. (1993) Am. J. Hematol. 42: 7-12).

Members of the cytochrome P450 family are also closely associated with vitamin D synthesis and catabolism. Vitamin D exists as two biologically

equivalent prohormones, ergocalciferol (vitamin D_2), produced in plant tissues and cholecalciferol (vitamin D_3), produced in animal tissues. The latter form, cholecalciferol, is formed upon the exposure of 7-dehydrocholesterol to near ultraviolet light (i.e., 290-310 nm), normally resulting from even minimal periods of skin exposure to sunlight (reviewed in Miller, W. L. and Portale, A. A. (2000) Trends in Endocrinology and Metabolism 11: 315-319).

Both prohormone forms are further metabolized in the liver to 25-hydroxyvitamin D (25(OH)D) by the enzyme 25-hydroxylase. 25(OH)D is the most abundant precursor form of vitamin D which must be further metabolized in the kidney to the active form, 1α,25-dihydroxyvitamin D (1α,25$(OH)_2$D), by the enzyme 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase). Regulation of 1α,25$(OH)_2$D production is primarily at this final step in the synthetic pathway. The activity of 1α-hydroxylase depends upon several physiological factors including the circulating level of the enzyme product (1α,25$(OH)_2$D) and the levels of parathyroid hormone (PTH), calcitonin, insulin, calcium, phosphorus, growth hormone, and prolactin. Furthermore, extrarenal 1α-hydroxylase activity has been reported, suggesting that tissue-specific, local regulation of 1α,25$(OH)_2$D production may also be biologically important. The catalysis of 1α,25$(OH)_2$D to 24,25-dihydroxyvitamin D (24,25$(OH)_2$D), involving the enzyme 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase), also occurs in the kidney. 24-hydroxylase

can also use $25(OH)_2D$ as a substrate (Shinki, T. *et al.* (1997) Proc. Natl. Acad. Sci. U. S. A. 94: 12920-12925 ; Miller, W. L. and Portale, A. A. supra; and references within).

Vitamin D 25-hydroxylase, 1 α-hydroxylase, and 24-hydroxylase are all NADPH-dependent, type I (mitochondrial) cytochrome P450 enzymes that show a high degree of homology with other members of the family. Vitamin D 25-hydroxylase also shows a broad substrate specificity and may also perform 26-hydroxylation of bile acid intermediates and 25,26, and 27-hydroxylation of cholesterol (Dilworth, F. J. *et al.* (1995) J. Biol. Chem. 270: 16766-16774; Miller, W. L. and Portale, A. A. supra; and references within).

The active form of vitamin D (1 α,$25(OH)_2D$) is involved in calcium and phosphate homeostasis and promotes the differentiation of myeloid and skin cells. Vitamin D deficiency resulting from deficiencies in the enzymes involved in vitamin D metabolism (e.g., 1 α-hydroxylase) causes hypocalcemia, hypophosphatemia, and vitamin D-dependent (sensitive) rickets, a disease characterized by loss of bone density and distinctive clinical features, including bandy or bow leggedness accompanied by a waddling gait. Deficiencies in vitamin D 25-hydroxylase cause cerebrotendinous xanthomatosis, a lipid-storage disease characterized by the deposition of cholesterol and cholestanol in the Achilles' tendons, brain, lungs, and many other tissues. The disease presents with progressive neurologic dysfunction, including postpubescent cerebellar

ataxia, atherosclerosis, and cataracts. Vitamin D 25-hydroxylase deficiency does not result in rickets, suggesting the existence of alternative pathways for the synthesis of 25 (OH) D (Griffin, J. E. and Zerwekh, J. E. (1983) J. Clin. Invest. 72: 1190-1199; Gamblin, G. T. *et al.* (1985) J. Clin. Invest. 75: 954-960; and W. L. and Portale, A. A. supra).

Ferredoxin and ferredoxin reductase are electron transport accessory proteins which support at least one human cytochrome P450 species, cytochrome P450c27 encoded by the CYP27 gene (Dilworth, F. J. *et al.* (1996) Biochem. J. 320: 267-71). A *Streptomyces sriseus* cytochrome P450, CYP104D1, was heterologously expressed in *E. coli* and found to be reduced by the endogenous ferredoxin and ferredoxin reductase enzymes (Taylor, M. *et al.* (1999) Biochem. Biophys. Res. Commun. 263: 838-42), suggesting that many cytochrome P450 species may be supported by the ferredoxin/ferredoxin reductase pair. Ferredoxin reductase has also been found in a model drug metabolism system to reduce actinomycin D, an antitumor antibiotic, to a reactive free radical species (Flitter, W. D. and Mason, R. P. (1988) Arch. Biochem. Biophys. 267: 632-9).

FLAVIN-CONTAINING MONOOXYGENASE (FMO)

Flavin-containing monooxygenases (FMO) oxidize the nucleophilic nitrogen, sulfur, and phosphorus heteroatom of an exceptional range of substrates. Like cytochromes P450, FMOs are microsomal and use NADPH and O_2; there is also a great deal of substrate overlap with cytochromes

P450. The tissue distribution of FMOs includes liver, kidney, and lung.

There are five different known isoforms of FMO in mammals (FMO1, FM02, FM03, FMO4, and FMOS), which are expressed in a tissue-specific manner. The isoforms differ in their substrate specificities and other properties such as inhibition by various compounds and stereospecificity of reaction. FMOs have a 13 amino acid signature sequence, the components of which span the N-terminal two-thirds of the sequences and include the FAD binding region and the FATGY motif which has been found in many N-hydroxylating enzymes (Stehr; M. *et al.* (1998) Trends Biochem. Sci. 23: 56-57).

Specific reactions include oxidation of nucleophilic tertiary amines to N-oxides, secondary amines to hydroxylamines and nitrones, primary amines to hydroxylamines and oximes, and sulfur containing compounds and phosphines to S-and P-oxides. Hydrazines, iodides, selenides, and boron-containing compounds are also substrates. Although FMOs appear similar to cytochromes P450 in their chemistry, they can generally be distinguished from cytochromes P450 *in vitro* based on, for example, the higher heat lability of FMOs and the nonionic detergent sensitivity of cytochromes P450; however, use of these properties in identification is complicated by further variation among FMO isoforms with respect to thermal stability and detergent sensitivity.

FMOs play important roles in the metabolism of several drugs and xenobiotics. FMO (FM03 in liver) is

predominantly responsible for metabolizing (S)-nicotine to (S)-nicotine N-1'-oxide, which is excreted in urine. FMO is also involved in S-oxygenation of cimetidine, an H2-antagonist widely used for the treatment of gastric ulcers. Liver-expressed forms of FMO are not under the same regulatory control as cytochrome P450. In rats, for example, phenobarbital treatment leads to the induction of cytochrome P450, but the repression of FMO1.

Endogenous substrates of FMO include cysteamine, which is oxidized to the disulfide, cystamin, and trimethylamine (TMA), which is metabolized to trimethylamine N-oxide. TMA smells like rotting fish, and mutations in the FM03 isoform lead to large amounts of the malodorous free amine being excreted in sweat, urine, and breath. These symptoms have led to the designation fish-odor syndrome.

LYSYL OXIDASE

Lysyl oxidase (lysine 6-oxidase, LO) is a copper-dependent amine oxidase involved in the formation of connective tissue matrices by cross-linking collagen and elastin. LO is secreted as a N-glycosylated precursor protein of approximately 50 kDa and cleaved to the mature form of the enzyme by a metalloprotease, although the precursor form is also active. The copper atom in LO is involved in the transport of electron to and from oxygen to facilitate the oxidative deamination of lysine residues in these extracellular matrix proteins. While the coordination of copper is essential to LO activity,

insufficient dietary intake of copper does not influence the expression of the apoenzyme. However, the absence of the functional LO is linked to the skeletal and vascular tissue disorders that are associated with dietary copper deficiency. LO is also inhibited by a variety of semicarbazides, hydrazines, and amino nitrites, as well as heparin. Beta-aminopropionitrile is a commonly used inhibitor. LO activity is increased in response to ozone, cadmium, and elevated levels of hormones released in response to local tissue trauma, such as transforming growth factor-beta, platelet-derived growth factor, angiotensin II, and fibroblast growth factor. Abnormalities in LO activity has been linked to Menkes syndrome and occipital horn syndrome. Cytosolic forms of the enzyme have been implicated in abnormal cell proliferation (reviewed in Rucker, R. B. et al. (1998) Am. J. Clin. Nutr. 67: 996S-1002S and Smith-Mungo. L. I. and Kagan, H. M. (1998) Matrix Biol. 16: 387-398).

DIHYDROFOLATE REDUCTASES

Dihydrofolate reductases (DHFR) are ubiquitous enzymes that catalyze the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, an essential step in the *de novo* synthesis of glycine and purines as well as the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). The basic reaction is as follows:

7,8-dihydrofolate + NADPH → 5,6,7,8-tetrahydrofolate + $NADP^+$

The enzymes can be inhibited by a number of dihydrofolate analogs, including trimethroprim and methotrexate. Since an abundance of TMP is required for DNA synthesis, rapidly dividing cells require the activity of DHFR. The replication of DNA viruses (one example is herpesvirus) also requires high levels of DHFR activity. As a result, drugs that target DHFR have been used for cancer chemotherapy and to inhibit DNA virus replication. (For similar reasons, thymidylate synthetases are also target enzymes.) Drugs that inhibit DHFR are preferentially cytotoxic for rapidly dividing cells (or DNA virus-infected cells) but have no specificity, resulting in the indiscriminate destruction of dividing cells. Furthermore, cancer cells may become resistant to drugs such as methotrexate as a result of acquired transport defects or the duplication of one or more DHFR genes (Stryer, L (1988) Biochemistry. W. H Freeman and Co., Inc. New York. pp. 511-5619).

ALDO/KETO REDUCTASES

Aldo/keto reductases are monomeric NADPH-dependent oxidoreductases with broad substrate specificities (Bohren, K. M. *et al.* (1989) J. Biol. Chem. 264: 9547-51). These enzymes catalyze the reduction of carbonyl-containing compounds, including carbonyl-containing sugars and aromatic compounds, to the corresponding alcohols. Therefore, a variety of carbonyl-containing drugs and

xenobiotics are likely metabolized by enzymes of this class.

One known reaction catalyzed by a family member, aldose reductase, is the reduction of glucose to sorbitol, which is then further metabolized to fructose by sorbitol dehydrogenase. Under normal conditions, the reduction of glucose to sorbitol is a minor pathway. In hyperglycemic states, however, the accumulation of sorbitol is implicated in the development of diabetic complications. Members of this enzyme family are also highly expressed in some liver cancers (Cao, D. *et al.* (1998) J. Biol. Chem. 273: 11429-35).

ALCOHOL DEHYDROGENASES

Alcohol dehydrogenases (ADHs) oxidize simple alcohols to the corresponding aldehydes. ADH is a cytosolic enzyme, prefers the cofactor NAD+, and also binds zinc ion. Liver contains the highest levels of ADH, with lower levels in kidney, lung, and the gastric mucosa.

Known ADH isoforms are dimeric proteins composed of 40 kDa subunits. There are five known gene loci which encode these subunits (a, b, g, p, c), and some of the loci have characterized allelic variants (b"b2, b3, g1, g2). The subunits can form homodimers and heterodimers; the subunit composition determines the specific properties of the active enzyme. The holoenzymes have therefore been categorized as Class I (subunit compositions aa, ab, ag, bg, gg), Class II (pp), and Class III (cc). Class I ADH isozymes oxidize ethanol and other small aliphatic

alcohols, and are inhibited by pyrazole. Class II isozymes prefer longer chain aliphatic and aromatic alcohols, are unable to oxidize methanol, and are not inhibited by pyrazole. Class III isozymes prefer even longer chain aliphatic alcohols (five carbons and longer) and aromatic alcohols, and are not inhibited by pyrazole.

The short-chain alcohol dehydrogenases include a number of related enzymes with a variety of substrate specificities. Included in this group are the mammalian enzymes D-beta-hydroxybutyrate dehydrogenase, (R)-3-hydroxybutyrate dehydrogenase, 15- hydroxyprostaglandin dehydrogenase, NADPH-dependent carbonyl reductase, corticosteroid 11-beta-dehydrogenase, and estradiol 17-beta-dehydrogenase, as well as the bacterial enzymes acetoacetyl-CoA reductase, glucose 1- dehydrogenase, 3-beta-hydroxysteroid dehydrogenase, 20-beta-hydroxysteroid dehydrogenase, ribitol dehydrogenase, 3-oxoacyl reductase, 2, 3-dihydro-2, 3-dihydroxybenzoate dehydrogenase, sorbitol-6-phosphate 2-dehydrogenase, 7-alpha-hydroxysteroid dehydrogenase, cis-1, 2-dihydroxy-3, 4-cyclohexadiene-1-carboxylate dehydrogenase, cis-toluene dihydrodiol dehydrogenase, cis-benzene glycol dehydrogenase, biphenyl-2, 3-dihydro-2, 3-diol dehydrogenase, N-acylmannosamine 1- dehydrogenase, and 2-deoxy-D-gluconate 3-dehydrogenase (Krozowski, Z. (1994) J. Steroid Biochem. Mol. Biol. 51: 125-130; Krozowski, Z. (1992) Mol. Cell Endocrinol. 84: C25-31; and Marks, A. R. *et al.* (1992) J. Biol. Chem. 267: 15459-15463).

UDP GLUCURONYLTRANSFERASE

Members of the UDP glucuronyltransferase family (UGTs) catalyze the transfer of a glucuronic acid group from the cofactor uridine diphosphate-glucuronic acid (UDP-glucuronic acid) to a substrate. The transfer is generally to a nucleophilic heteroatom (O, N, or S). Substrates include xenobiotics which have been functionalized by Phase I reactions, as well as endogenous compounds such as bilirubin, steroid hormones, and thyroid hormones. Products of glucuronidation are excreted in urine if the molecular weight of the substrate is less than about 250 g/mol, whereas larger glucuronidated substrates are excreted in bile.

UGTs are located in the microsomes of liver, kidney, intestine, skin, brain, spleen, and nasal mucosa, where they are on the same side of the endoplasmic reticulum membrane as cytochrome P450 enzymes and flavin-containing monooxygenases, and therefore are ideally located to access products of Phase I drug metabolism. UGTs have a C-terminal membrane-spanning domain which anchors them in the endoplasmic reticulum membrane and a conserved signature domain of about 50 amino acid residues in their C terminal section.

UGTs involved in drug metabolism are encoded by two gene families, UGT1 and UGT2. Members of the UGT1 family result from alternative splicing of a single gene locus, which has a variable substrate binding domain and constant region involved in cofactor binding and membrane insertion. Members of the UGT2 family are encoded by

separate gene loci, and are divided into two families, UGT2A and UGT2B. The 2A subfamily is expressed in olfactory epithelium, and the 2B subfamily is expressed in liver microsomes. Mutations in UGT genes are associated with hyperbilirubinemia; Crigler-Najjar syndrome, characterized by intense hyperbilirubinemia from birth; and a milder form of hyperbilirubinemia termed Gilbert's disease.

SULFOTRANSFERASE

Sulfate conjugation occurs on many of the same substrates which undergo O-glucuronidation to produce a highly water-soluble sulfuric acid ester. Sulfotransferases (ST) catalyze this reaction by transferring SO_3-from the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the substrate. ST substrates are predominantly phenols and aliphatic alcohols, but also include aromatic amines and aliphatic amines, which are conjugated to produce the corresponding sulfamates. The products of these reactions are excreted mainly in urine.

STs are found in a wide range of tissues, including liver, kidney, intestinal tract, lung, platelets, and brain. The enzymes are generally cytosolic, and multiple forms are often co-expressed. For example, there are more than a dozen forms of ST in rat liver cytosol. These biochemically characterized STs fall into five classes based on their substrate preference: arylsulfotransferase, alcohol sulfotransferase, estrogen

sulfotransferase, tyrosine ester sulfotransferase, and bile salt sulfotransferase.

ST enzyme activity varies greatly with sex and age in rats. The combined effects of developmental cues and sex-related hormones are thought to lead to these differences in ST expression profiles, as well as the profiles of other DMEs such as cytochromes P450. Notably, the high expression of STs in cats partially compensates for their low level of UDP glucuronyltransferase activity.

Several forms of ST have been purified from human liver cytosol and cloned. There are two phenol sulfotransferases with different thermal stabilities and substrate preferences. The thermostable enzyme catalyzes the sulfation of phenols such as para-nitrophenol, minoxidil, and acetaminophen; the thermolabile enzyme prefers monoamine substrates such as dopamine, epinephrine, and levadopa. Other cloned STs include an estrogen sulfotransferase and an N-acetylglucosamine-6-O-sulfotransferase. This last enzyme is illustrative of the other major role of STs in cellular biochemistry, the modification of carbohydrate structures that may be important in cellular differentiation and maturation of proteoglycans. Indeed, an inherited defect in a sulfotransferase has been implicated in macular corneal dystrophy, a disorder characterized by a failure to synthesize mature keratan sulfate proteoglycans (Nakazawa, K. *et al.* (1984) J. Biol. Chem. 259: 13751-7).

GALACTOSYLTRANSFERASES

Galactosyltransferases are a subset of glycosyltransferases that transfer galactose (Gal) to the terminal N-acetylglucosamine (GlcNAc) oligosaccharide chains that are part of glycoproteins or glycolipids that are free in solution (Kolbinger, F. *et al.* (1998) J. Biol. Chem. 273: 433-440; Amado, M. *et al.* (1999) Biochim. Biophys. Acta 1473: 35-53). Galactosyltransferases have been detected on the cell surface and as soluble extracellular proteins, in addition to being present in the Golgi. β1,3-galactosyltransferases form Type I carbohydrate chains with Gal (β1-3) GlcNAc linkages. Known human and mouse β1,3-galactosyltransferases appear to have a short cytosolic domain, a single transmembrane domain, and a catalytic domain with eight conserved regions. (Kolbinger, F. supra and Hennet, T. *et al.* (1998) J. Biol. Chem. 273: 58-65). In mouse UDP-galactose : β-N-acetylglucosamine β1,3-galactosyltransferase-I region 1 is located at amino acid residues 78-83, region 2 is located at amino acid residues 93-102, region 3 is located at amino acid residues 116-119, region 4 is located at amino acid residues 147-158, region 5 is located at amino acid residues 172-183, region 6 is located at amino acid residues 203-206, region 7 is located at amino acid residues 236-246, and region 8 is located at amino acid residues 264-275. A variant of a sequence found within mouse UDP-galactose:β-N-acetylglucosamine β1,3-galactosyltransferase-I region 8 is also found in bacterial galactosyltransferases, suggesting that this

sequence defines a galactosyltransferase sequence motif (Hennet, T. supra). Recent work suggests that brainiac protein is a β1,3-galactosyltransferase. (Yuan, Y. *et al.* (1997) Cell 88: 9-11; and Hennet, T. supra).

UDP-Gal:GlcNAc-1, 4-galactosyltransferase (-1, 4-GalT) (Sato, T. *et al.*, (1997) EMBO J. 16: 1850-1857) catalyzes the formation of Type II carbohydrate chains with Gal (β1-4) GlcNAc linkages. As is the case with the β1,3-galactosyltransferase, a soluble form of the enzyme is formed by cleavage of the membrane-bound form. Amino acids conserved among β1,4-galactosyltransferases include two cysteines linked through a disulfide-bonded and a putative UDPgalactose-binding site in the catalytic domain (Yadav, S. and Brew, K. (1990) J. Biol. Chem. 265: 14163-14169; Yadav, S. P. and Brew, K. (1991) J. Biol. Chem. 266: 698-703; and Shaper, N. L. *et al.* (1997) J. Biol. Chem. 272: 31389-31399). β1,4-galactosyltransferases have several specialized roles in addition to synthesizing carbohydrate chains on glycoproteins or glycolipids. In mammals a β1,4-galactosyltransferase, as part of a heterodimer with cc-lactalbumin, functions in lactating mammary gland lactose production. A β1,4-galactosyltransferase on the surface of sperm functions as a receptor that specifically recognizes the egg. Cell surface β1,4-galactosyltransferases also function in cell adhesion, cell/basal lamina interaction, and normal and metastatic cell migration (Shur, B. (1993) Curr. Opin. Cell Biol. 5: 854-863; and Shaper, J. (1995) Adv. Exp. Med. Biol. 376: 95-104).

GLUTATHIONE S-TRANSFERASE

The basic reaction catalyzed by glutathione S-transferases (GST) is the conjugation of an electrophile with reduced glutathione (GSH). GSTs are homodimeric or heterodimeric proteins localized mainly in the cytosol, but some level of activity is present in microsomes as well. The major isozymes share common structural and catalytic properties; in humans they have been classified into four major classes, Alpha, Mu, Pi, and Theta. The two largest classes, Alpha and Mu, are identified by their respective protein isoelectric points; pI ~ 7.5-9.0 (Alpha), and pI ~ 6.6 Mu). Each GST possesses a common binding site for GSH and a variable hydrophobic binding site. The hydrophobic binding site in each isozyme is specific for particular electrophilic substrates. Specific amino acid residues within GSTs have been identified as important for these binding sites and for catalytic activity. Residues Q67, T68, D101, E104, and R131 are important for the binding of GSH (Lee, H-C *et al.* (1995) J. Biol. Chem. 270: 99-109). Residues R13, R20, and R69 are important for the catalytic activity of GST (Stenberg G *et al.* (1991) Biochem. J. 274: 549-55).

In most cases, GSTs perform the beneficial function of deactivation and detoxification of potentially mutagenic and carcinogenic chemicals. However, in some cases their action is detrimental and results in activation of chemicals with consequent mutagenic and carcinogenic effects. Some forms of rat and human GSTs are

reliable preneoplastic markers that aid in the detection of carcinogenesis. Expression of human GSTs in bacterial strains, such as *Salmonella typhimurium* used in the well-known Ames test for mutagenicity, has helped to establish the role of these enzymes in mutagenesis. Dihalomethanes, which produce liver tumors in mice, are believed to be activated by GST. This view is supported by the finding that dihalomethanes are more mutagenic in bacterial cells expressing human GST than in untransfected cells (Thier, R. *et al.* (1993) Proc. Natl. Acad. Sci. USA 90: 8567-80). The mutagenicity of ethylene dibromide and ethylene dichloride is increased in bacterial cells expressing the human Alpha GST, A1-1, while the mutagenicity of allatoxin B1 is substantially reduced by enhancing the expression of GST (Simula, T. P. *et al.* (1993) Carcinogenesis 14: 1371-6). Thus, control of GST activity may be useful in the control of mutagenesis and carcinogenesis.

GST has been implicated in the acquired resistance of many cancers to drug treatment, the phenomenon known as multi-drug resistance (MDR). MDR occurs when a cancer individual is treated with a cytotoxic drug such as cyclophosphamide and subsequently becomes resistant to this drug and to a variety of other cytotoxic agents as well. Increased GST levels are associated with some of these drug resistant cancers, and it is believed that this increase occurs in response to the drug agent which is then deactivated by the GST catalyzed GSH conjugation reaction. The increased GST levels then protect the cancer cells from other cytotoxic agents which bind to GST. Increased levels of A1-1 in tumors has been linked to drug

resistance induced by cyclophosphamide treatment (Dirven H. A. et al. (1994) Cancer Res. 54: 6215-20). Thus control of GST activity in cancerous tissues may be useful in treating MDR in cancer individuals.

GAMMA-GLUTAMYL TRANSPEPTIDASE

Gamma-glutamyl transpeptidases are ubiquitously expressed enzymes that initiate extracellular glutathione (GSH) breakdown by cleaving gamma-glutamyl amide bonds. The breakdown of GSH provides cells with a regional cysteine pool for biosynthetic pathways. Gamma-glutamyl transpeptidases also contribute to cellular antioxidant defenses and expression is induced by oxidative stress. The cell surface-localized glycoproteins are expressed at high levels in cancer cells. Studies have suggested that the high level of gamma-glutamyl transpeptidases activity present on the surface of cancer cells could be exploited to activate precursor drugs, resulting in high local concentrations of anticancer therapeutic agents (Hanigan, M. H. (1998) Chem. Biol. Interact. 111-112: 333-42 ; Taniguchi, N. and Ikeda, Y. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72: 239-78 ; Chikhi, N. et al. (1999) Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 122: 367-80).

ACYLTRANSFERASE

N-acyltransferase enzymes catalyze the transfer of an amino acid conjugate to an activated carboxylic group.

Endogenous compounds and xenobiotics are activated by acyl-CoA synthetases in the cytosol, microsomes, and mitochondria. The acyl-CoA intermediates are then conjugated with an amino acid (typically glycine, glutamin, or taurine, but also ornithine, arginine, histidine, serine, aspartic acid, and several dipeptides) by N-acyltransferases in the cytosol or mitochondria to form a metabolite with an amide bond. This reaction is complementary to O-glucuronidation, but amino acid conjugation does not produce the reactive and toxic metabolites which often result from glucuronidation.

One well-characterized enzyme of this class is the bile acid-CoA : amino acid N-acyltransferase (BAT) responsible for generating the bile acid conjugates which serve as detergents in the gastrointestinal tract (Falany, C. N. et al. (1994) J. Biol. Chem. 269: 19375-9 ; Johnson, M. R. et al. (1991) J. Biol. Chem. 266: 10227-33). BAT is also useful as a predictive indicator for prognosis of hepatocellular carcinoma individuals after partial hepatectomy (Furutani, M. et al. (1996) Hepatology 24: 1441-5).

ACETYLTRANSFERASES

Acetyltransferases have been extensively studied for their role in histone acetylation. Histone acetylation results in the relaxing of the chromatin structure in eukaryotic cells, allowing transcription factors to gain access to promoter elements of the DNA templates in the affected region of the genome (or the genome in general).

In contrast, histone deacetylation results in a reduction in transcription by closing the chromatin structure and limiting access of transcription factors. To this end, a common means of stimulating cell transcription is the use of chemical agents that inhibit the deacetylation of histones (e. g., sodium butyrate), resulting in a global (albeit artifactual) increase in gene expression. The modulation of gene expression by acetylation also results from the acetylation of other proteins, including but not limited to, p53, GATA-1, MyoD, ACTR, TFIIE, TFIIF and the high mobility group proteins (HMG). In the case of p53, acetylation results in increased DNA binding, leading to the stimulation of transcription of genes regulated by p53. The prototypic histone acetylase (HAT) is Gcn5 from *Saccharomyces cerevisiae*. Gcn5 is a member of a family of acetylases that includes Tetrahymena p55, human GcnS, and human p300/CBP. Histone acetylation is reviewed in (Cheung, W. L. et al. (2000) Current Opinion in Cell Biology 12 : 326-333 and Berger, S. L (1999) Current Opinion in Cell Biology 11 : 336-341). Some acetyltransferase enzymes posses the alpha/beta hydrolase fold common to several other major classes of enzymes, including but not limited to, acetylcholinesterases and carboxylesterases.

N-ACETYLTRANSFERASE

Aromatic amines and hydrazine-containing compounds are subject to N-acetylation by the N-acetyltransferase enzymes of liver and other tissues. Some xenobiotics can

be O-acetylated to some extent by the same enzymes. N-acetyltransferases are cytosolic enzymes which utilize the cofactor acetyl-coenzyme A (acetyl-CoA) to transfer the acetyl group in a two step process. In the first step, the acetyl group is transferred from acetyl-CoA to an active site cysteine residue; in the second step, the acetyl group is transferred to the substrate amino group and the enzyme is regenerated.

In contrast to most other DME classes, there are a limited number of known N-acetyltransferases. In humans, there are two highly similar enzymes, NAT1 and NAT2; mice appear to have a third form of the enzyme, NAT3. The human forms of N-acetyltransferase have independent regulation (NAT1 is widely-expressed, whereas NAT2 is in liver and gut only) and overlapping substrate preferences. Both enzymes appear to accept most substrates to some extent, but NAT1 does prefer some substrates (para-aminobenzoic acid, para-aminosalicylic acid, sulfamethoxazole, and sulfanilamide), while NAT2 prefers others (isoniazid, hydralazine, procainamide, dapsone, aminoglutethimide, and sulfamethazine).

Clinical observations of individuals taking the antituberculosis drug isoniazid in the 1950s led to the description of fast and slow acetylators of the compound. These phenotypes were shown subsequently to be due to mutations in the NAT2 gene which affected enzyme activity or stability. The slow isoniazid acetylator phenotype is very prevalent in Middle Eastern populations (approx. 70%), and is less prevalent in Caucasian (approx. 50%) and

Asian (<25%) populations. More recently, functional polymorphism in NAT1 has been detected, with approximately 8% of the population tested showing a slow acetylator phenotype (Butcher, N. J. *et al.* (1998) Pharmacogenetics 8: 67-72). Since NAT1 can activate some known aromatic amine carcinogens, polymorphism in the widely-expressed NAT1 enzyme may be important in determining cancer risk.

AMINOTRANSFERASES

Aminotransferases comprise a family of pyridoxal 5'-phosphate (PLP)-dependent enzymes that catalyze transformations of amino acids. Aspartate aminotransferase (AspAT) is the most extensively studied PLP-containing enzyme. It catalyzes the reversible transamination of dicarboxylic L-amino acids, aspartate and glutamate, and the corresponding 2-oxo acids, oxaloacetate and 2-oxoglutarate. Other members of the family included pyruvate aminotransferase, branched-chain amino acid aminotransferase, tyrosine aminotransferase, aromatic aminotransferase, alanine : glyoxylate aminotransferase (AGT), and kynurenine aminotransferase (Vacca, R. A. *et al.* (1997) J. Biol. Chem.272: 21932-21937).

Primary hyperoxaluria type-1 is an autosomal recessive disorder resulting in a deficiency in the liver-specific peroxisomal enzyme, alanine : glyoxylate aminotransferase-1. The phenotype of the disorder is a deficiency in glyoxylate metabolism. In the absence of AGT, glyoxylate is oxidized to oxalate rather than being transaminated to glycine. The result is the deposition of

insoluble calcium oxalate in the kidneys and urinary tract, ultimately causing renal failure (Lumb, M. J. *et al.* (1999) J. Biol. Chem. 274: 20587-20596).

Kynurenine aminotransferase catalyzes the irreversible transamination of the L-tryptophan metabolite L-kynurenine to form kynurenic acid. The enzyme may also catalyze the reversible transamination reaction between L-2-aminoadipate and 2-oxoglutarate to produce 2-oxoadipate and L-glutamate. Kynurenic acid is a putative modulator of glutamatergic neurotransmission, thus a deficiency in kynurenine aminotransferase may be associated with pleotrophic effects (Buchli, R. *et al.* (1995) J. Biol. Chem. 270: 29330-29335).

CATECHOL-O-METHYLTRANSFERASE

Catechol-O-methyltransferase (COMT) catalyzes the transfer of the methyl group of S-adenosylmethionine (AdoMet ; SAM) donor to one of the hydroxyl groups of the catechol substrate (e.g., L-dopa, dopamine, or DBA). Methylation of the 3'-hydroxyl group is favored over methylation of the 4'-hydroxyl group and the membrane bound isoform of COMT is more regiospecific than the soluble form. Translation of the soluble form of the enzyme results from utilization of an internal start codon in a full-length mRNA (1.5 kb) or from the translation of a shorter mRNA (1.3 kb), transcribed from an internal promoter. The proposed S_N2-like methylation reaction requires Mg^{2+} and is inhibited by Ca^{2+}. The binding of the donor and substrate to COMT occurs sequentially. AdoMet

first binds COMT in a Mg^{2+}-independent manner, followed by the binding of Mg^{2+} and the binding of the catechol substrate.

The amount of COMT in tissues is relatively high compared to the amount of activity normally required, thus inhibition is problematic. Nonetheless, inhibitors have been developed for *in vitro* use (e.g., galates, tropolone, U-0521, and 3',4'-dihydroxy-2-methyl-propiophetropolone) and for clinical use (e.g., nitrocatechol-based compounds and tolcapone). Administration of these inhibitors results in the increased half-life of L-dopa and the consequent formation of dopamine. Inhibition of COMT is also likely to increase the half-life of various other catechol-structure compounds, including but not limited to epinephrine/norepinephrine, isoprenaline, rimiterol, dobutamine, fenoldopam, apomorphine, and α-methyldopa. A deficiency in norepinephrine has been linked to clinical depression, hence the use of COMT inhibitors could be useful in the treatment of depression. COMT inhibitors are generally well tolerated with minimal side effects and are ultimately metabolized in the liver with only minor accumulation of metabolites in the body (Männistö, P. T. and Kaakkola, S. (1999) Pharmacological Reviews 51: 593-628).

COPPER-ZINC SUPEROXIDE DISMUTASES

Copper-zinc superoxide dismutases are compact homodimeric metalloenzymes involved in cellular defenses against oxidative damage. The enzymes contain one atom of

zinc and one atom of copper per subunit and catalyze the dismutation of superoxide anions into O_2 and H_2O_2. The rate of dismutation is diffusion-limited and consequently enhanced by the presence of favorable electrostatic interactions between the substrate and enzyme active site. Examples of this class of enzyme have been identified in the cytoplasm of all the eukaryotic cells as well as in the periplasm of several bacterial species. Copper-zinc superoxide dismutases are robust enzymes that are highly resistant to proteolytic digestion and denaturing by urea and SDS. In addition to the compact structure of the enzymes, the presence of the metal ions and intrasubunit disulfide bonds is believed to be responsible for enzyme stability. The enzymes undergo reversible denaturation at temperatures as high as 70°C (Battistoni, A. *et al.* (1998) J. Biol. Chem. 273:655-5661).

Overexpression of superoxide dismutase has been implicated in enhancing freezing tolerance of transgenic Alfalfa as well as providing resistance to environmental toxins such as the diphenyl ether herbicide, acifluorfen (McKersie, B. D. *et al.* (1993) Plant Physiol. 103: 1155-1163). In addition, yeast cells become more resistant to freeze-thaw damage following exposure to hydrogen peroxide which causes the yeast cells to adapt to further peroxide stress by upregulating expression of superoxide dismutases. In this study, mutations to yeast superoxide dismutase genes had a more detrimental effect on freeze-thaw resistance than mutations which affected the regulation of glutathione metabolism, long suspected of

being important in determining an organism's survival through the process of cryopreservation (Jong-In Park, J-I. *et al.* (1998) J. Biol. Chem. 273: 22921-22928).

Expression of superoxide dismutase is also associated with *Mycobacterium tuberculosis*, the organism that causes tuberculosis. Superoxide dismutase is one of the ten major proteins secreted by *M. tuberculosis* and its expression is upregulated approximately 5-fold in response to oxidative stress. *M. tuberculosis* expresses almost two orders of magnitude more superoxide dismutase than the nonpathogenic mycobacterium *M. smegmatis*, and secretes a much higher proportion of the expressed enzyme. The result is the secretion of 350-fold more enzyme by *M. tuberculosis* than *M. smegmatis*, providing substantial resistance to oxidative stress (Harth, G. and Horwitz, M. A. (1999) J. Biol. Chem. 274: 4281-4292).

The reduced expression of copper-zinc superoxide dismutases, as well as other enzymes with anti-oxidant capabilities, has been implicated in the early stages of cancer. The expression of copper-zinc superoxide dismutases has been shown to be lower in prostatic intraepithelial neoplasia and prostate carcinomas, compared to normal prostate tissue (Bostwick, D. G. (2000) Cancer 89: 123-134).

PHOSPHODIESTERASES

Phosphodiesterases make up a class of enzymes which catalyze the hydrolysis of one of the two ester bonds in a phosphodiester compound. Phosphodiesterases are

therefore crucial to a variety of cellular processes. Phosphodiesterases include DNA and RNA endonucleases and exonucleases, which are essential for cell growth and replication, and topoisomerases, which break and rejoin nucleic acid strands during topological rearrangement of DNA. A Tyr-DNA phosphodiesterase functions in DNA repair by hydrolyzing dead-end covalent intermediates formed between topoisomerase I and DNA (Pouliot, J. J. *et al.* (1999) Science 286: 552-555; Yang, S.-W. (1996) Proc. Natl. Acad. Sci. USA 93: 11534-11539).

Acid sphingomyelinase is a phosphodiesterase which hydrolyzes the membrane phospholipid sphingomyelin to produce ceramide and phosphorylcholine. Phosphorylcholine is used in the synthesis of phosphatidylcholine, which is involved in numerous intracellular signaling pathways, while ceramide is an essential precursor for the generation of gangliosides, membrane lipids found in high concentration in neural tissue. Defective acid sphingomyelinase leads to a build-up of sphingomyelin molecules in lysosomes, resulting in Niemann-Pick disease (Schuchman, E. H. and S. R. Miranda (1997) Genet. Test. 1: 13-19).

Glycerophosphoryl diester phosphodiesterase (also known as glycerophosphodiester phosphodiesterase) is a phosphodiesterase which hydrolyzes deacetylated phospholipid glycerophosphodiesters to produce sn-glycerol-3-phosphate and an alcohol. Glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoglycerol, and glycerophosphoinositol are

examples of substrates for glycerophosphoryl diester phosphodiesterases. A glycerophosphoryl diester phosphodiesterase from *E. coli* has broad specificity for glycerophosphodiester substrates (Larson, T. J. *et al.* (1983) J. Biol. Chem. 248: 5428-5432).

Cyclic nucleotide phosphodiesterases (PDEs) are crucial enzymes in the regulation of the cyclic nucleotides cAMP and cGMP. cAMP and cGMP function as intracellular second messengers to transduce a variety of extracellular signals including hormones, light, and neurotransmitters. PDEs degrade cyclic nucleotides to their corresponding monophosphates, thereby regulating the intracellular concentrations of cyclic nucleotides and their effects on signal transduction. Due to their roles as regulators of signal transduction, PDEs have been extensively studied as chemotherapeutic targets (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-81; Torphy, J. T. (1998) Am. J. Resp. Crit. CareMed. 157: 351-370).

Families of mammalian PDEs have been classified based on their substrate specificity and affinity, sensitivity to cofactors, and sensitivity to inhibitory agents (Beavo, J. A. (1995) Physiol. Rev. 75: 725-748; Conti, M. *et al.* (1995) Endocrine Rev. 16: 370-389). Several of these families contain distinct genes, many of which are expressed in different tissues as splice variants. Within PDE families, there are multiple isozymes and multiple splice variants of these isozymes (Conti, M. and S. L. C. Jin (1999) Prog. Nucleic Acid Res. Mol. Biol.

63: 1-38). The existence of multiple PDE families, isozymes, and splice variants is an indication of the variety and complexity of the regulatory pathways involving cyclic nucleotides (Houslay, M. D. and G. Milligan (1997) Trends Biochem. Sci. 22: 217224).

Type 1 PDEs (PDE1s) are Ca^{2+}/calmodulin-dependent and appear to be encoded by at least three different genes, each having at least two different splice variants (Kakkar, R. *et al.* (1999) Cell Mol. Life Sci. 55: 1164-1186). PDE1s have been found in the lung, heart, and brain. Some PDE1 isozymes are regulated *in vitro* by phosphorylation/dephosphorylation. Phosphorylation of these PDE1 isozymes decreases the affinity of the enzyme for calmodulin, decreases PDE activity, and increases steady state levels of cAMP (Kakkar, *supra*). PDE1s may provide useful therapeutic targets for disorders of the central nervous system, and the cardiovascular and immune systems due to the involvement of PDE1s in both cyclic nucleotide and calcium signaling (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481).

PDE2s are cGMP-stimulated PDEs that have been found in the cerebellum, neocortex, heart, kidney, lung, pulmonary artery, and skeletal muscle (Sadhu, K. et al. (1999) J. Histochem. Cytochem. 47: 895-906). PDE2s are thought to mediate the effects of cAMP on catecholamine secretion, participate in the regulation of aldosterone (Beavo, *supra*), and play a role in olfactory signal transduction (Juilfs, D. M. *et al.* (1997) Proc. Natl. Acad. Sci. USA 94: 3388-3395).

PDE3s have high affinity for both cGMP and cAMP, and so these cyclic nucleotides act as competitive substrates for PDE3s. PDE3s play roles in stimulating myocardial contractility, inhibiting platelet aggregation, relaxing vascular and airway smooth muscle, inhibiting proliferation of T-lymphocytes and cultured vascular smooth muscle cells, and regulating catecholamine-induced release of free fatty acids from adipose tissue. The PDE3 family of phosphodiesterases are sensitive to specific inhibitors such as cilostamide, enoximone, and lixazinone. Isozymes of PDE3 can be regulated by cAMP-dependent protein kinase, or by insulin-dependent kinases (Degerman, E. *et al.* (1997) J. Biol. Chem. 272: 6823-6826).

PDE4s are specific for cAMP, are localized to airway smooth muscle, the vascular endothelium, and all inflammatory cells; and can be activated by cAMP-dependent phosphorylation. Since elevation of cAMP levels can lead to suppression of inflammatory cell activation and to relaxation of bronchial smooth muscle, PDE4s have been studied extensively as possible targets for novel anti-inflammatory agents, with special emphasis placed on the discovery of asthma treatments. PDE4 inhibitors are currently undergoing clinical trials as treatments for asthma, chronic obstructive pulmonary disease, and atopic eczema. All four known isozymes of PDE4 are susceptible to the inhibitor rolipram, a compound which has been shown to improve behavioral memory in mice (Barad, M. *et al.* (1998) Proc. Natl. Acad. Sci. USA 95: 15020-15025). PDE4 inhibitors have also been studied as possible therapeutic agents against acute lung injury, endotoxemia, rheumatoid

arthritis, multiple sclerosis, and various neurological and gastrointestinal indications (Doherty, A. M. (1999) Curr. Opin. Chem. Biol. 3: 466-473).

PDE5 is highly selective for cGMP as a substrate (Turko, I. V. *et al.* (1998) Biochemistry 37: 4200-4205), and has two allosteric cGMP-specific binding sites (McAllister-Lucas, L. M. *et al.* (1995) J. Biol. Chem. 270: 30671-30679). Binding of cGMP to these allosteric binding sites seems to be important for phosphorylation of PDE5 by cGMP-dependent protein kinase rather than for direct regulation of catalytic activity. High levels of PDE5 are found in vascular smooth muscle, platelets, lung, and kidney. The inhibitor zaprinast is effective against PDE5 and PDEls. Modification of zaprinast to provide specificity against PDE5 has resulted in sildenafil (VIAGRA; Pfizer, Inc., New York NY), a treatment for male erectile dysfunction (Terrett, N. *et al.* (1996) Bioorg. Med. Chem. Lett. 6: 1819-1824). Inhibitors of PDE5 are currently being studied as agents for cardiovascular therapy (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481).

PDE6s, the photoreceptor cyclic nucleotide phosphodiesterases, are crucial components of the phototransduction cascade. In association with the G-protein transducin, PDE6s hydrolyze cGMP to regulate cGMP-gated cation channels in photoreceptor membranes. In addition to the cGMP-binding active site, PDE6s also have two high-affinity cGMP-binding sites which are thought to play a regulatory role in PDE6 function (Artemyev, N. O.

et al. (1998) Methods 14: 93-104). Defects in PDE6s have been associated with retinal disease. Retinal degeneration in the rd mouse (Yan, W. *et al.* (1998) Invest. Opthalmol. Vis. Sci. 39: 2529-2536), autosomal recessive retinitis pigmentosa in humans (Danciger, M. *et al.* (1995) Genomics 30: 1-7), and rod/cone dysplasia 1 in Irish Setter dogs (Suber, M. L. *et al.* (1993) Proc. Natl. Acad. Sci. USA 90: 3968- 972) have been attributed to mutations in the PDE6B gene.

The PDE7 family of PDEs consists of only one known member having multiple splice variants (Bloom, T. J. and J. A. Beavo (1996) Proc. Natl. Acad. Sci. USA 93: 14188-14192). PDE7s are cAMP specific, but little else is known about their physiological function. Although mRNAs encoding PDE7s are found in skeletal muscle, heart, brain, lung, kidney, and pancreas, expression of PDE7 proteins is restricted to specific tissue types (Han, P. *et al.* (1997) J. Biol. Chem. 272: 16152-16157 ; Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481). PDE7s are very closely related to the PDE4 family; however, PDE7s are not inhibited by rolipram, a specific inhibitor of PDE4s (Beavo, *supra*).

PDE8s are cAMP specific, and are closely related to the PDE4 family. PDE8s are expressed in thyroid gland, testis, eye, liver, skeletal muscle, heart, kidney, ovary, and brain. The cAMP hydrolyzing activity of PDE8s is not inhibited by the PDE inhibitors rolipram, vinpocetine, milrinone, IBMX (3-isobutyl-1-methylxanthine), or zaprinast, but PDE8s are inhibited by dipyridamole

(Fisher, D. A. *et al.* (1998) Biochem. Biophys. Res. Commun. 246: 570-577 ; Hayashi, M. *et al.* (1998) Biochem. Biophys. Res. Commun. 250: 751-756 ; Soderling, S. H. *et al.* 1998) Proc. Natl. Acad. Sci. USA 95: 8991-8996).

PDE9s are cGMP specific and most closely resemble the PDE8 family of PDEs. PDE9s are expressed in kidney, liver, lung, brain, spleen, and small intestine. PDE9s are not inhibited by sildenafil (VIAGRA; Pfizer, Inc., New York NY), rolipram, vinpocetine, dipyridamole, or IBMX (3-isobutyl-1methylxanthine), but they are sensitive to the PDE5 inhibitor zaprinast (Fisher, D. A. *et al.* (1998) J. Biol. Chem. 273: 15559-15564 ; Soderling, S. H. *et al.* (1998) J. Biol. Chem. 273: 15553-15558).

PDE10s are dual-substrate PDEs, hydrolyzing both cAMP and cGMP. PDE10s are expressed in brain, thyroid, and testis. (Soderling, S. H. *et al.* (1999) Proc. Natl. Acad. Sci. USA 96: 7071-7076; Fujishige, K. *et al.* (1999) J. Biol. Chem. 274: 18438-18445; Loughney, K. *et al.* (1999) Gene 234: 109117).

PDEs are composed of a catalytic domain of about 270-300 amino acids, an N-terminal regulatory domain responsible for binding cofactors, and, in some cases, a hydrophilic C-terminal domain of unknown function (Conti, M. and S.-L. C. Jin (1999) Prog. Nucleic Acid Res. Mol. Biol. 63: 1-38). A conserved, putative zinc-binding motif, HDXXHXGXXN, has been identified in the catalytic domain of all PDEs. N-terminal regulatory domains include non-catalytic cGMP-binding domains in PDE2s, PDE5s, and PDE6s; calmodulin-binding domains in PDE1s; and domains

containing phosphorylation sites in PDE3s and PDE4s. In PDE5, the N-terminal cGMP-binding domain spans about 380 amino acid residues and comprises tandem repeats of the conserved sequence motif N (R/K) XnFX3DE (McAllister-Lucas, L. M. *et al.* (1993) J. Biol. Chem. 268: 22863-22873). The NKXnD motif has been shown by mutagenesis to be important for cGMP binding (Turko, I. V. *et al.* (1996) J. Biol. Chem. 271: 22240-22244). PDE families display approximately 30% amino acid identity within the catalytic domain; however, isozymes within the same family typically display about 85-95% identity in this region (e.g. PDE4A vs PDE4B). Furthermore, within a family there is extensive similarity (>60%) outside the catalytic domain; while across families, there is little or no sequence similarity outside this domain.

Many of the constituent functions of immune and inflammatory responses are inhibited by agents that increase intracellular levels of cAMP (Verghese, M. W. *et al.* (1995) Mol. Pharmacol. 47: 1164-1171). A variety of diseases have been attributed to increased PDE activity and associated with decreased levels of cyclic nucleotides. For example, a form of diabetes insipidus in mice has been associated with increased PDE4 activity, an increase in low-K_m cAMP PDE activity has been reported in leukocytes of atopic individuals, and PDE3 has been associated with cardiac disease.

Many inhibitors of PDEs have been identified and have undergone clinical evaluation (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481; Torphy,

T. J. (1998) Am. J. Respir. Crit. Care Med. 157: 351-370). PDE3 inhibitors are being developed as antithrombotic agents, antihypertensive agents, and as cardiotonic agents useful in the treatment of congestive heart failure. Rolipram, a PDE4 inhibitor, has been used in the treatment of depression, and other inhibitors of PDE4 are undergoing evaluation as anti-inflammatory agents. Rolipram has also been shown to inhibit lipopolysaccharide (LPS) induced TNF-a, which has been shown to enhance HIV-1 replication in vitro. Therefore, rolipram may inhibit HIV-1 replication (Angel, J. B. *et al.* (1995) AIDS 9: 1137-1144). Additionally, rolipram, based on its ability to suppress the production of cytokines such as TNF-a, and b and interferon g, has been shown to be effective in the treatment of encephalomyelitis. Rolipram may also be effective in treating tardive dyskinesia and was effective in treating multiple sclerosis in an experimental animal model (Sommer, N. *et al.* (1995) Nat. Med. 1: 244-248; Sasaki, H. *et al.* (1995) Eur. J. Pharmacol. 282: 71-76).

Theophylline is a nonspecific PDE inhibitor used in the treatment of bronchial asthma and other respiratory diseases. Theophylline is believed to act on airway smooth muscle function and in an anti-inflammatory or immunomodulatory capacity in the treatment of respiratory diseases (Banner, K. H. and C. P. Page (1995) Eur. Respir. J. 8: 996-1000). Pentoxifylline is another nonspecific PDE inhibitor used in the treatment of intermittent claudication and diabetes-induced peripheral vascular disease. Pentoxifylline is also known to block TNF-a

production and may inhibit HIV-1 replication (Angel *et al.*, *supra*).

PDEs have been reported to affect cellular proliferation of a variety of cell types (Conti *et al.* (1995) Endocrine Rev. 16: 370-389) and have been implicated in various cancers. Growth of prostate carcinoma cell lines DU145 and LNCaP was inhibited by delivery of cAMP derivatives and PDE inhibitors (Bang, Y. J. *et al.* (1994) Proc. Natl. Acad. Sci. USA 91: 5330-5334). These cells also showed a permanent conversion in phenotype from epithelial to neuronal morphology. It has also been suggested that PDE inhibitors have the potential to regulate mesangial cell proliferation (Matousovic, K. *et al.* (1995) J. Clin. Invest. 96: 401-410) and lymphocyte proliferation (Joulain, C. *et al.* (1995) J. Lipid Mediat. Cell Signal. 11: 63-79). A cancer treatment has been described that involves intracellular delivery of PDEs to particular cellular compartments of tumors, resulting in cell death (Deonarain, M. P. and A. A. Epenetos (1994) Br. J. Cancer 70: 786-794).

PHOSPHOTRIESTERASES

Phosphotriesterases (PTE, paraoxonases) are enzymes that hydrolyze toxic organophosphorus compounds and have been isolated from a variety of tissues. The enzymes appear to be lacking in birds and insects, but is abundant in mammals, explaining the reduced tolerance of birds and insects to organophosphorus compound (Vilanova, E. and Sogorb, M. A. (1999) Crit. Rev. Toxicol. 29: 21-

57). Phosphotriesterases play a central role in the detoxification of insecticides by mammals. Phosphotriesterase activity varies among individuals and is lower in infants than adults. Knockout mice are markedly more sensitive to the organophosphate-based toxins diazoxon and chlorpyrifos oxon (Furlong, C. E., *et al.* (2000) Neurotoxicology 21: 91-100). PTEs have attracted interest as enzymes capable of the detoxification of organophosphate-containing chemical waste and warfare reagents (e.g., parathion), in addition to pesticides and insecticides. Some studies have also implicated phosphotriesterase in atherosclerosis and diseases involving lipoprotein metabolism.

THIOESTERASES

Two soluble thioesterases involved in fatty acid biosynthesis have been isolated from mammalian tissues, one which is active only toward long-chain fatty-acyl thioesters and one which is active toward thioester with a wide range of fatty-acyl chain-lengths. These thioesterases catalyze the chain-terminating step in the *de novo* biosynthesis of fatty acids. Chain termination involves the hydrolysis of the thioester bond which links the fatty acyl chain to the 4'-phosphopantetheine prosthetic group of the acyl carrier protein (ACP) subunit of the fatty acid synthase (Smith, S. (1981a) Methods Enzymol. 71: 181-188; Smith, S. (1981b) Methods Enzymol. 71: 188-200).

E. coli contains two soluble thioesterases, thioesterase I (TEI) which is active only toward long-chain acyl thioesters, and thioesterase II (TEII) which has a broad chain-length specificity (Naggert, J. *et al.* (1991) J. Biol. Chem. 266: 11044-11050). *E. coli* TEII does not exhibit sequence similarity with either of the two types of mammalian thioesterases which function as chain-terminating enzymes in *de novo* fatty acid biosynthesis. Unlike the mammalian thioesterases, *E. coli* TEII lacks the characteristic serine active site gly-X-ser-X-gly sequence motif and is not inactivated by the serine modifying agent diisopropyl fluorophosphate. However, modification of histidine 58 by iodoacetamide and diethylpyrocarbonate abolished TEII activity. Overexpression of TEII did not alter fatty acid content in *E. coli*, which suggests that it does not function as a chain-terminating enzyme in fatty acid biosynthesis (Naggert *et al.*, *supra*). For that reason, Naggert *et al.* (*supra*) proposed that the physiological substrates for *E. coli* TEII may be coenzyme A (CoA)-fatty acid esters instead of ACP-phosphopanthetheine-fatty acid esters.

CARBOXYLESTERASES

Mammalian carboxylesterases constitute a multigene family expressed in a variety of tissues and cell types. Isozymes have significant sequence homology and are classified primarily on the basis of amino acid sequence. Acetylcholinesterase, butyrylcholinesterase, and carboxylesterase are grouped into the serine super family

of esterases (B-esterases). Other carboxylesterases included thyroglobulin, thrombin, Factor IX, gliotactin, and plasminogen. Carboxylesterases catalyze the hydrolysis of ester and amide-groups from molecules and are involved in detoxification of drugs, environmental toxins, and carcinogens. Substrates for carboxylesterases include short-and long-chain acyl-glycerols, acylcarnitine, carbonates, dipivefrin hydrochloride, cocaine, salicylates, capsaicin, palmitoyl-coenzyme A, imidapril, haloperidol, pyrrolizidine alkaloids, steroids, p-nitrophenyl acetate, malathion, butanilicaine, and isocarboxazide. The enzymes often demonstrate low substrate specificity. Carboxylesterases are also important for the conversion of prodrugs to their respective free acids, which may be the active form of the drug (e.g., lovastatin, used to lower blood cholesterol) (reviewed in Satoh, T. and Hosokawa, M. (1998) Annu. Rev. Pharmacol. Toxicol. 38: 257-288).

Neuroligins are a class of molecules that (i) have N-terminal signal sequences, (ii) resemble cell-surface receptors, (iii) contain carboxylesterase domains, (iv) are highly expressed in the brain, and (v) bind to neurexins in a calcium-dependent manner. Despite the homology to carboxylesterases, neuroligins lack the active site serine residue, implying a role in substrate binding rather than catalysis (Ichtchenko, K. *et al.* (1996) J. Biol. Chem. 271: 2676-2682).

SQUALENE EPOXIDASE

Squalene epoxidase (squalene monooxygenase, SE) is a microsomal membrane-bound, FAD-dependent oxidoreductase that catalyzes the first oxygenation step in the sterol biosynthetic pathway of eukaryotic cells. Cholesterol is an essential structural component of cytoplasmic membranes acquired via the LDL receptor-mediated pathway or the biosynthetic pathway. In the latter case, all 27 carbon atoms in the cholesterol molecule are derived from acetyl-CoA (Stryer, L., *supra*). SE converts squalene to 2, 3 (S)-oxidosqualene, which is then converted to lanosterol and then cholesterol. The steps involved in cholesterol biosynthesis are summarized below (Stryer, L (1988) Biochemistry. W. H Freeman and Co., Inc. New York. pp. 554-560 and Sakakibara, J. *et al.* (1995) 270: 17-20) :

acetate (from Acetyl-CoA) → 3-hydoxy-3-methyl-glutaryl CoA → mevalonate → 5-phosphomevalonate → 5-pyrophosphomevalonate → isopentenyl pyrophosphate → dimethylallyl pyrophosphate → geranyl pyrophosphate → farnesyl pyrophosphate → squalene → squalene epoxide → lanosterol → cholesterol.

While cholesterol is essential for the viability of eukaryotic cells, inordinately high serum cholesterol levels results in the formation of atherosclerotic plaques in the arteries of higher organisms. This deposition of highly insoluble lipid material onto the walls of essential blood vessels (e.g., coronary arteries) results

in decreased blood flow and potential necrosis of the tissues deprived of adequate blood flow. HMG-CoA reductase is responsible for the conversion of 3-hydroxyl-3-methylglutaryl CoA (HMG-CoA) to mevalonate, which represents the first committed step in cholesterol biosynthesis. HMG-CoA is the target of a number of pharmaceutical compounds designed to lower plasma cholesterol levels. However, inhibition of MHG-CoA also results in the reduced synthesis of non-sterol intermediates (e.g., mevalonate) required for other biochemical pathways. SE catalyzes a rate-limiting reaction that occurs later in the sterol synthesis pathway and cholesterol is the only end product of the pathway following the step catalyzed by SE. As a result, SE is the ideal target for the design of anti-hyperlipidemic drugs that do not cause a reduction in other necessary intermediates (Nakamura, Y. *et al.* (1996) 271: 8053-8056).

EPOXIDE HYDROLASES

Epoxide hydrolases catalyze the addition of water to epoxide-containing compounds, thereby hydrolyzing epoxides to their corresponding 1, 2-diols. They are related to bacterial haloalkane dehalogenases and show sequence similarity to other members of the α/β hydrolase fold family of enzymes (e.g., bromoperoxidase A2 from *Streptomyces aureofaciens*, hydroxymuconic semialdehyde hydrolases from *Pseudomonas putida*, and haloalkane dehalogenase from *Xanthobacter autotrophicus*). Epoxide hydrolases are ubiquitous in nature and have been found in

mammals, invertebrates, plants, fungi, and bacteria. This family of enzymes is important for the detoxification of xenobiotic epoxide compounds which are often highly electrophilic and destructive when introduced into an organism. Examples of epoxide hydrolase reactions include the hydrolysis of cis-9, 10-epoxyoctadec-9 (Z)-enoic acid (leukotoxin) to form its corresponding diol, threo-9, 10-dihydroxyowtadec-12 (Z)-enoic acid (leukotoxin diol), and the hydrolysis of cis-12, 13-epoxyoctadec-9 (Z)-enoic acid (isoleukotoxin) to form its corresponding diol threo-12, 13-dihydroxyoctadec-9 (Z)-enoic acid (isoleukotoxin diol). Leukotoxins alter membrane permeability and ion transport and cause inflammatory responses. In addition, epoxide carcinogens are known to be produced by cytochrome P450 as intermediates in the detoxification of drugs and environmental toxins.

The enzymes possess a catalytic triad composed of Asp (the nucleophile), Asp (the histidine-supporting acid), and His (the water-activating histidine). The reaction mechanism of epoxide hydrolase proceeds via a covalently bound ester intermediate initiated by the nucleophilic attack of one of the Asp residues on the primary carbon atom of the epoxide ring of the target molecule, leading to a covalently bound ester intermediate (Michael Arand, M. *et al.* (1996) J. Biol. Chem. 271: 4223-4229; Rink, R. *et al.* (1997) J. Biol. Chem. 272: 14650-14657; Argiriadi, M. A. *et al.* (2000) J. Biol. Chem. 275: 15265-15270).

ENZYMES INVOLVED IN TYROSINE CATALYSIS

The degradation of the amino acid tyrosine to either succinate and pyruvate or fumarate and acetoacetate, requires a large number of enzymes and generates a large number of intermediate compounds. In addition, many xenobiotic compounds may be metabolized using one or more reactions that are part of the tyrosine catabolic pathway. While the pathway has been studied primarily in bacteria, tyrosine degradation is known to occur in a variety of organisms and is likely to involve many of the same biological reactions.

The enzymes involved in the degradation of tyrosine to succinate and pyruvate (e.g., in *Artlirobacter* species) include 4-hydroxyphenylpyruvate oxidase, 4-hydroxyphenylacetate 3-hydroxylase, 3, 4-dihydroxyphenylacetate 2, 3-dioxygenase, 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase, trans, cis-5-carboxymethyl-2-hydroxymuconate isomerase, homoprotocatechuate isomerase/decarboxylase, cis-2-oxohept-3-ene-1, 7-dioate hydratase, 2, 4-dihydroxyhept-trans-2-ene-1, 7-dioate aldolase, and succinic semialdehyde dehydrogenase.

The enzymes involved in the degradation of tyrosine to fumarate and acetoacetate (e.g., in *Pseudontonas* species) include 4-hydroxyphenylpyruvate dioxygenase, homogentisate 1, 2-dioxygenase, maleylacetoacetate isomerase, and fumarylacetoacetase. 4-hydroxyphenylacetate 1-hydroxylase may also be involved if

intermediates from the succinate/pyruvate pathway are accepted.

Additional enzymes associated with tyrosine metabolism in different organisms include 4-chlorophenylacelate-3, 4-dioxygenase, aromatic aminotransferase, 5-oxopent-3-ene-1, 2, 5-tricarboxylate decarboxylase, 2-oxo-hept-3-ene-1, 7-dioate hydratase, and 5-carboxymethyl-2-hydroxymuconate isomerase (Ellis, L. B. M. *et al.* (1999) Nucleic Acids Res. 27: 373-376; Wackett, L. P. and Ellis, L. B. M. (1996) J. Microbiol. Meth. 25: 91-93; and Schmidt, M. (1996) Amer. Soc. Microbiol. News 62: 102).

In humans, acquired or inherited genetic defects in enzymes of the tyrosine degradation pathway may result in hereditary tyrosinemia. One form of this disease, hereditary tyrosinemia 1 (HT1) is caused by a deficiency in the enzyme fumarylacetoacetate hydrolase, the last enzyme in the pathway in organisms that metabolize tyrosine to fumarate and acetoacetate. HT1 is characterized by progressive liver damage beginning at infancy, and increased risk for liver cancer (Endo, F. *et al.* (1997) J. Biol. Chem. 272: 24426-24432).

An enzyme of one system can act on several drugs and drug metabolites. The rate of metabolism of a drug differs between individuals and between ethnic groups, owing to the existence of enzymatic polymorphism within each system. Metabolic phenotypes have been generally characterized as poor metabolizers (PM), extensive metabolizers (EM), and ultra-extensive metabolizers (UEM).

Knowledge of a metabolic phenotype is clinically useful for the following reasons:

1) a phenotype may be correlated to an individual's susceptibility to toxic chemicals, diseases and cancers;

2) a phenotype may provide a physician with valuable information for quickly determining a safe and therapeutically-effective drug treatment regimen for an individual; and

3) individual phenotypes may provide valuable rationales for the design of therapeutic drugs.

To date, the ability to characterize multiple phenotypic determinants for the purpose of identifying individual phenotypes, drug treatment compatibility and susceptibility has been limited by the complexities of multiple metabolic pathways, and the lack of efficient and effective procedures for making these determinations. Currently, the determination of an individual's phenotype for a given metabolic enzyme can be performed either via direct metabolic phenotyping or indirect extrapolation of an individual's genotype to a given phenotype.

Direct phenotyping involves the use a probe substrate known to be metabolized by a given enzyme. The rate of metabolism of the probe substrate is measured and this rate of metabolism is used to determine a metabolic phenotype. Although labor intensive and costly procedures for direct phenotyping have been known for many years these procedures are not readily adaptable for a clinical

environment, nor are they practical for measuring multiple phenotypic determinants. For example, enzymatic phenotypes may be determined by measurements of the molar (or chiral) ratio of metabolites of a drug or a probe substrate in a urine sample from a individual by high-pressure liquid chromatography (HPLC), capillary electrophoresis (CE) or stereo-selective capillary gas chromatography. These determination methods are time-consuming, onerous, and employ systems and equipment that are not readily available in a clinical laboratory. Methodologies for the rapid determination of multiple determinants of a metabolic phenotypic are not available, and as a result, valuable information concerning an individual's phenotype is not considered on a routine basis in a clinical environment.

Indirect phenotyping can be defined as assigning a phenotype based on non-functional measurements. These non-functional measurements include genotyping, haplotyping, gene expression and protein expression analysis. The patent application, WO 00/63683 provides an extensive description of various methods developed to perform the aforementioned analysis.

Genotyping is performed by analyzing the genetic sequence of a gene coding for a specific enzyme by a polymerase chain reaction assay (PCR) or a PCR with a restriction fragment length polymorphism assay (PCR-RFLP). The gene is examined for the presence of genetic mutations that can be linked to increased or decreased enzyme levels or activity, which in turn result in a specific phenotype,

i.e. a slow metabolizer vs. a fast metabolizer. The genotype is a theoretical measurement of what an individual's phenotype should be. Haplotyping is an extension of genotyping in which the genotype of different gene alleles are considered. For example if a person had one wild type (wt) gene sequence and one mutant (mt) gene sequence, the individual would have a wt/mt haplotype. Gene expression and protein expression analysis is defined as the measurement of mRNA/cDNA and protein levels respectively.

Indirect phenotyping may be limited by several factors that can result in an alteration in the theoretical phenotype. For example it has been well established that genotype does not always correlate with phenotype, likewise gene expression does not always correlate with protein expression, and protein expression does not always correlate with protein function. Indirect phenotyping fails to account for many factors that affect protein function including but not limited to post-translational protein modification, polypharmacy, and exposure to inducers or inhibitors. Furthermore, other limitations include the potential complexity of performing a complete genotyping. The mutation sequence must first be identified before they can be examined in a genotyping assay. Subsequent to identification, the mutation must be linked to a definitive effect on phenotype. For some enzymes, there appear to be very few mutations and those found have been well characterized, while for other enzymes multiple mutations are present with new mutations being found regularly (e.g. CYP2D6 has over 53 mutations

and 48 allelic variants). Therefore, while genotyping for CYP2C19 might be performed with relatively few measurements, a complete and accurate genotyping of CYP2D6 would be complex and require multiple measurements.

Indirect phenotyping suffers from complexity and the direct phenotyping techniques are not easily accessible to clinical settings,

Physicians routinely prescribe treatment regimes without knowledge of an individual's metabolic capability (phenotype) or genotype for metabolism. Accordingly, a trial and error treatment regime is initiated, often at the expense of severe side effects and loss of valuable treatment time.

The need for a method to predict an individual's response to a drug therapy (both efficacy of therapy and occurrence of side effects) has been recognized by many in the field. The importance of drug metabolizing can be explained as follows. If inhibition of a particular system leads to toxicity, then low gene or protein expression of components of this system might be used to identify individuals with high risk of toxicity. Likewise those individual's with high expression levels would be considered to be at low risk. However, if the individual classified as a low risk individual, also has low metabolism of the drug, then the drug will remain in the system much longer and may have the time to eliminate the function of the system which as a result leads to toxicity. Conversely, if an individual has low system activity but is also a rapid drug metabolizer, than it is

possible that there will not be sufficient drug present at any given point to induce toxicity by inhibiting the system. Therefore, the knowledge of an individual's drug metabolizing capabilities is an essential component of individualized drug therapy.

The ability to rapidly and accurately identify multiple metabolic phenotypic determinants on an individual basis would provide a physician with valuable individual-specific information that could be readily applied in selecting a safe and effective treatment regime for that individual. Similarly, knowledge of multi-determinant metabolic phenotypics would also find valuable application in research and drug development. In particular, individual phenotypes could be identified prior to a drug treatment trial. Moreover, knowledge of multi-determinant metabolic phenotypes would have applications in the development of new drugs, so-called rational drug design.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a method for selecting an individual treatment regime.

Accordingly, another aim of the present invention is to provide a method for the individualization of treatment with an anticoagulant.

Yet another aim of the present invention is to provide a method for selecting candidates for clinical treatment trials.

Still another aim of the present invention is to provide a method of using multi-determinant phenotyping for the individualization of treatment with anticoagulants.

In accordance with one aspect of the present invention, there is provided a method of characterizing a multi-determinant metabolic phenotype for at least one anticoagulant agent, wherein a plurality of phenotypic determinants are identified as corresponding to respective metabolic characteristics; said method comprising: a) administering to an individual a probe substrate specific to metabolic pathway(s) for said at least one anticoagulant agent; b) detecting metabolites of said metabolic pathway(s) in a biological sample from said individual in response to said probe substrate; and c) characterizing respective phenotypic determinants of said multi-determinant metabolic phenotype based on detected metabolites.

In accordance with yet another aspect of the present invention, there is provided a method of using a multi-determinant metabolic phenotype to individualize a treatment regimen for at least one anticoagulant agent for an individual, wherein the multi-determinant metabolic phenotype of said individual is determined; a safe and therapeutically effective dose of said at least one anticoagulant agent treatment is determined and/or selected based on said multi-determinant metabolic phenotype of said individual.

In accordance with yet a further aspect of the invention, there is provided a method of administering to an individual at least one anticoagulant agent, said method comprising: a) determining a multi-determinant metabolic phenotype of said individual; and administering a safe and therapeutically effective dose of said at least one anticoagulant agent to said individual, wherein said dose has been determined based on a metabolic profile of said individual corresponding to said individual's metabolic phenotype for said at least one anticoagulant agent as represented by said multi-determinant metabolic phenotype.

In accordance with still a further aspect of the invention, there is provided an assay system for detecting the presence of enzyme-specific metabolites in a biological sample, said sample obtained from an individual treated with a known amount of at least one probe substrate for at least one anticoagulant agent, specific for metabolic pathways of said metabolites, said assay comprising: a) means for receiving said biological sample, including a plurality of affinity complexation agents contained therein; b) means for detecting presence of said enzyme-specific metabolites bound to said affinity complexation agents; and c) means for quantifying ratios of said metabolites to provide corresponding phenotypic determinants; wherein said phenotypic determinants provide a metabolic phenotypic profile of said individual.

In accordance with yet another aspect of the present invention there is provided a method of using an

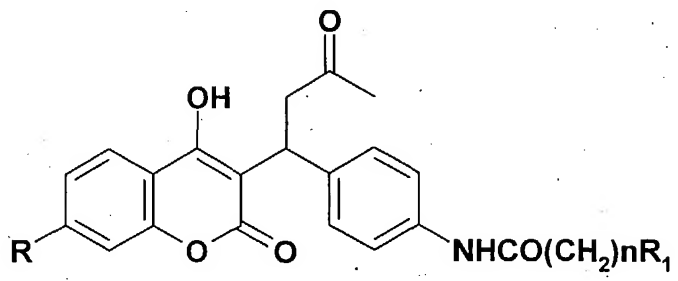
enzyme-specific assay for the individualization of treatment with at least one anticoagulant agent, said method comprising: a) conducting said assay to identify phenotypic determinants in a biological sample obtained from an individual treated with a probe substrate for said at least one anticoagulant agent; b) determining a rate of drug metabolism according to said determinants; and c) determining and/or selecting a safe and therapeutically effective dose of said class of anticoagulant agents for said individual based on said rate.

In accordance with yet another aspect of the present invention there is provided a method of screening a plurality of individuals for participation in a drug treatment trial assessing the therapeutic effect of at least one anticoagulant agent, said method comprising: selecting individuals having a metabolic phenotype characterized as effective for metabolizing said at least one anticoagulant agent.

In accordance with yet another aspect of the present invention there is provided a method of screening a plurality of individuals for treatment with at least one anticoagulant agent, said method comprising: a) genotyping said individuals to identify individuals lacking at least one allelic variation known to prompt toxicity of said at least one anticoagulant agent; and b) selecting individuals having a metabolic phenotype characterized as effective for metabolizing said at least one anticoagulant agent.

In accordance with yet another aspect of the present invention there is provided a method of screening a plurality of individuals for participation in a drug treatment trial assessing the therapeutic effect of a candidate anticoagulant agent treatment, said method comprising: a) genotyping each of said individuals to identify individuals lacking at least one allelic variation known to prompt the toxicity of said anticoagulant agent; and b) characterizing a multi-determinant metabolic phenotype of said identified individuals of step a) to determine each individual's ability to metabolize said anticoagulant agent.

In accordance with yet another aspect of the present invention there are provided compounds having formula V:



wherein, n = 1-8, R is H, or OH and R_1 is selected from the group consisting of CO_2H, OH, NH_2 and CH_2X, wherein X is a halogen.

In accordance with yet another aspect of the present invention there is provided an immunogenic composition for raising antibodies specific to warfarin in a subject, which comprises a compound of the present invention

modified with an immunogenic moiety or carrier in association with a pharmaceutically acceptable carrier.

In accordance with yet another aspect of the present invention there is provided a method of raising antibodies which bind to warfarin, which comprises administering an immunogenic amount of an immunogenic composition of the present invention to an animal.

In accordance with yet another aspect of the present invention there is provided a method of producing antibodies which bind to warfarin, comprising: a) treating an animal with an immunogenic amount of an immunogenic composition of the present invention to produce antibodies; and b) isolating the antibodies of step a) from serum of the animal.

In accordance with yet another aspect of the present invention there is provided an isolated antibody or antigen binding fragment thereof, which binds to a compound of the present invention.

In accordance with yet another aspect of the present invention there is provided a hybridoma cell line that produces a monoclonal antibody which binds to a compound of the present.invention.

In accordance with yet another aspect of the present invention there is provided an assay kit for detecting the presence of enzyme-specific metabolites in a biological sample, the sample obtained from an individual treated with a known amount of at least one probe substrate for at least one anticoagulant agent, which comprises at least two antibodies each specific to the at

least one probe substrate or a different metabolite of the at least one probe substrate to measure their molar ratio in a biological sample of an individual after being treated with the at least one probe substrate, wherein the at least one antibody is an antibody according to the present invention.

For the purpose of the present invention the following terms are defined below.

The term "phenotypic determinant" is intended to mean a qualitative or quantitative indicator of an enzyme-specific capacity of an individual.

The term "individualization" as it appears herein with respect to therapy is intended to mean a therapy having specificity to at least an individual's phenotype as calculated according to a predetermined formula on an individual basis.

The term "biological sample" is intended to mean a sample obtained from a biological entity and includes, but is not to be limited to, any one of the following: tissue, cerebrospinal fluid, plasma, serum, saliva, blood, nasal mucosa, urine, synovial fluid, microcapillary microdialysis and breath.

The term "anticoagulant agent" is intended to mean any agent which interferes with the clotting of blood. Some anticoagulants, such as the coumarin derivatives bishydroxycoumarin (Dicumarol) and warfarin (Coumadin) inhibit synthesis of prothrombin, a clot-forming substance, and other clotting factors. Anticoagulants can include but are not limited to compounds acting as beta2

Adrenoreceptor Antagonists, Neuropeptide V2 Antagonists, prostacyclin analogs, thromboxane synthase inhibitors, calcium agonists, coumarin derivatives, elastase inhibitors, Non-steroidal antiinflammatories thrombin inhibitors, lipoxygenase inhibitors, Factor VIIa inhibitors, Factor Xa inhibitors, phosphodiesterase III inhibitors, Heparins, and fibrinogen glucoprotein IIb/IIIa Antagonists.

The term "treatment" is intended to mean any administration of a pharmaceutical compound to an individual to treat, cure, alleviate, improve, diminish or inhibit a condition in the individual, including, without limitation, administering anticoagulants to interfere with thrombosis in an individual.

The term "individual treated" is intended to mean any individual being subjected to the administration of i) a pharmaceutical compound, for treating, curing, alleviating, improving, diminishing or inhibiting a condition, or ii) a probe substrate for determining multi-determinant metabolic phenotype.

The term "condition" is intended to mean any condition causing coagulation of blood in an individual, including atherosclerosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates metabolites of the CYP3A4 enzymatic pathway according to an embodiment of the present invention;

Fig. 2 illustrates metabolites of the NAT2 enzymatic pathway according to an embodiment of the present invention;

Fig. 3 illustrates metabolites of the CYP1A2 enzymatic pathway according to another embodiment of the present invention;

Fig. 4 illustrates metabolites of the NAT1 enzymatic pathway according to another embodiment of the present invention;

Fig. 5 illustrates metabolites of the CYP2A6 enzymatic pathway according to another embodiment of the present invention;

Fig. 6 illustrates metabolites of the CYP2C19 enzymatic pathway according to another embodiment of the present invention;

Fig. 7 illustrates metabolites of the CYP2C9 enzymatic pathway according to another embodiment of the present invention;

Fig. 8 illustrates metabolites of the CYP2D6 enzymatic pathway according to another embodiment of the present invention;

Fig. 9 illustrates metabolites of the CYP2E1 enzymatic pathway according to another embodiment of the present invention;

Fig. 10 illustrates the Vitamin K Cycle and its Inhibition by Warfarin;

Fig. 11 illustrates the scheme of the general immunosensor design depicting the intimate integration of

immunological recognition at the solid-state surface and the signal transduction;

Fig. 12 illustrates the principle of SPR technology;

Fig. 13 illustrates a TSM immunosensor device;

Fig. 14 illustrates the synthetic routes for the production of AAMU and 1X derivatives used in accordance with one embodiment of the present invention;

Figs. 15 to 18 show other AAMU and 1X derivatives which can be used for raising antibodies in accordance with another embodiment of the present invention;

Fig. 19 illustrates the absorbance competitive antigen ELISA curves of AAMU-Ab and 1X-Ab in accordance with one embodiment of the present invention;

Fig. 20 is a histogram of molar ratio of AAMU/1X;

Fig. 21 illustrates an ELISA array in accordance with an embodiment of the present invention;

Fig. 22 illustrates an ELISA array in accordance with another embodiment of the present invention;

Fig. 23 illustrates an ELISA detection system in accordance with another embodiment of the present invention.

Fig. 24 illustrates an assay system in accordance with another embodiment of the present invention;

Fig. 25 illustrates the general scheme of the synthesis of novel warfarin derivatives of the present invention;

Fig. 26 illustrates individualized dosing schemes for direct vs. indirect phenotyping in accordance with yet another embodiment of the present invention; and

Fig. 27 illustrates the clustering of International Normalization Ratio (INR) values for individuals treated with warfarin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the individualization of drug treatment. In particular, the present invention relates to the individualization of drug treatment with anticoagulants. Based on a phenotypic characterization of an individual's capacity to metabolize cytochrome P450-specific anticoagulants, the present invention provides a system and method for determining a dosage of an anticoagulant on an individual basis. The primary anticoagulant in use is warfarin. Warfarin used in the clinic is a racemic mixture of (S)- and (R)-warfarin, with (S)-warfarin responsible for the majority of it's anticoagulant activity. (S)-warfarin is primarily metabolized by the CYP2C9 enzyme, while (R)-Warafrin is also metabolized by the CYP3A4 enzyme. The present invention provides a method for quickly and accurately determining phenotypic determinants for the CYP2C9 metabolic pathway that can be used to characterize an individual's CYP2C9 specific phenotype. In doing so, a characterization of an individual's ability to metabolize an anticoagulant drug can be made and a corresponding drug dosage specific for that individual can be determined.

Further, the present invention provides a method for determining multiple phenotypic determinants that can be used to characterize a phenotypic profile of an individual that will exemplify that individual's ability to metabolize a given drug or group of drugs. Although most drugs are metabolized by a primary enzymatic pathway, such as CYP2C9 metabolizes (S)-warfarin, it is often the case that a given drug may be metabolized by multiple enzymes, such as (R)-Warfarin metabolized by CYP2C9 and CYP3A4. As a result, it may be preferred to characterize an individual's phenotypic profile for a plurality of metabolic enzymes prior to selecting a corresponding drug treatment regime. Knowledge of an individual's metabolic phenotype may be applied clinically in determining a phenotype-specific drug dosage based on the individual's capacity to metabolize the drug. Other factors representing an individual's capacity to metabolize a drug may also find application in the present invention, together with a phenotypic profile for obtaining individualization of therapy.

Accordingly, a system of the present invention is exemplified in accordance with a protocol for determining phenotypic determinants for NAT2. This protocol is adapted to provide a system for determining phenotypic determinants for at least CYP2C9 in accordance with the present invention. The determination of metabolic determinants for CYP2C9 may be performed as a single determination. Additionally, the determination of metabolic determinants may be performed with methods of determining a phenotypic profile for at least one of the

following enzymes: NAT1, NAT2, CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP2C19 and CYP3A4 the metabolites of which are illustrated in Figs. 1-9. These enzymes are involved in the metabolism of a large number of drugs, and as a result have important implications in the outcome of individual drug treatment regimes, and hence, clinical trial studies. These enzymes and their corresponding phenotypic determinants as described herein are provided as a representative example of determinants for the purposes of exemplifying the multi-determinant metabolic phenotyping of the present invention. However, the present invention is not limited thereto.

The present invention provides the ability to identify multiple phenotypic determinants of these enzymatic pathways for use in the individualization of drug treatment with anticoagulants.

ANTICOAGULANTS

THE THROMBOTIC PROCESS AND ITS COMPLICATIONS

Thrombi are composed of fibrin and blood cells and may form in any part of the cardiovascular system, including the veins, arteries, heart, and microcirculation. Because the relative proportions of cells and fibrin depend on hemodynamic factors, they differ in arterial and venous thrombi. Arterial thrombi form under conditions of high flow and are composed mainly of platelet aggregates bound together by thin fibrin

strands. In contrast, venous thrombi form in areas of stasis and are composed mainly of red cells with a large amount of interspersed fibrin and relatively few platelets. Thrombi that form in regions of slow to moderate flow are composed of a mixture of red cells, platelets, and fibrin and are known as mixed platelet-fibrin thrombi. When a platelet-rich arterial thrombus becomes occlusive, stasis occurs and the thrombus can propagate as a red stasis thrombus.

As thrombi age, they undergo progressive structural changes. Leukocytes are attracted by chemotactic factors released from aggregated platelets or proteolytic fragments of plasma proteins and become incorporated into the thrombi. The aggregated platelets swell and disintegrate and are gradually replaced by fibrin. Eventually the fibrin clot is digested by fibrinolytic enzymes released from endothelial cells and leukocytes or becomes organized by connective tissue.

The complications of thrombosis are caused by the effects of local obstruction of the vessel, distant embolization of thrombotic material, or, less commonly, consumption of hemostatic elements by their participation in the thrombotic process.

Arterial thrombi usually form either in regions of disturbed flow or at sites of rupture of atherosclerotic plaques. Plaque rupture exposes the thrombogenic subendothelium to platelets and coagulation proteins; it may also cause further narrowing due to hemorrhage into the plaque. Arterial thrombi may remain partially

occlusive or they may embolize. Nonocclusive thrombi may become incorporated into the vessel wall and can accelerate the growth of atherosclerotic plaques. When flow is slow, the degree of stenosis severe, or the thrombogenic stimulus intense, the thrombi may become totally occlusive. Arterial thrombi usually occur in association with preexisting vascular disease, the most common of which is atherosclerosis; they produce clinical manifestations by inducing tissue ischemia, either by obstructing flow or by embolizing into the distal microcirculation. Activation of blood coagulation as well as platelet activation is important in the pathogenesis of arterial thrombosis. These two fundamental mechanisms of thrombogenesis are closely linked *in vivo*, because thrombin, a key clotting enzyme generated by blood coagulation, is a potent platelet activator, and activated platelets augment the coagulation process. Therefore, both anticoagulants and drugs that suppress platelet function are potentially effective in the prevention and treatment of arterial thrombosis, and their benefit has been demonstrated by the results of clinical trials.

Venous thrombi usually occur in the lower limbs and are often asymptomatic; however, they can produce acute symptoms if they cause inflammation of the vessel wall, obstruct flow, or embolize into the pulmonary circulation. They can produce long-term complications due to venous hypertension if they damage the venous valves. Activation of blood coagulation is the critical mechanism in pathogenesis of venous thromboembolism, while the role of platelet activation is less important. Therefore, as

might be anticipated, anticoagulants are very effective for the prevention and treatment of venous thromboembolism, while drugs that suppress platelet function are of less benefit.

Intracardiac thrombi usually form on inflamed or damaged valves, on endocardium adjacent to a region of myocardial infarction, in a dilated or dyskinetic cardiac chamber, or on prosthetic valves. They are usually asymptomatic when confined to the heart but may produce serious complications if they embolize to the brain or the systemic circulation. Activation of blood coagulation appears to be more important in the pathogenesis of intracardiac thrombi than platelet activation, although the latter process also plays a contributory role. Anticoagulants are effective for prevention and treatment of intracardiac thrombi, and there is evidence that for individuals with prosthetic heart valves the efficacy of anticoagulants is augmented by drugs that suppress platelet function.

Widespread microvascular thrombosis is a complication of disseminated intravascular coagulation or generalized platelet aggregation. By blocking blood flow to the tissues, microthrombi can produce ischemic damage. In addition, red cell fragmentation can occur as the cells traverse the clot-filled vessels, leading to a hemolytic anemia. Finally, activation of the coagulation system can lead to a hemorrhagic disorder because of consumption of platelets and clotting factors. Anticoagulants are

effective in selected cases of disseminated intravascular coagulation.

CLINICAL CONSEQUENCES OF THROMBOSIS AND NEED FOR ANTICOAGULANTS

It has been estimated that venous thromboembolism is responsible for more than 300,000 hospital admissions per year in the United States. Pulmonary embolism causes or contributes to death in approximately 12% of individuals who are in hospitals and has been estimated to be responsible for 50,000 to 250,000 deaths per year in the United States.

The burden of venous thromboembolism is due to death from pulmonary embolism, the long-term consequences of the postthrombotic syndrome, the need for hospitalization, the complications of anticoagulant therapy, and the psychological effects of having a potentially recurrent and chronic illness.

Thrombosis is responsible for many of the acute manifestations of atherosclerosis and contributes to its progression. The effect of atherosclerosis is enormous. As a generalized pathological process, atherosclerosis affects the arteries to the heart, brain, abdomen, and legs, causing acute and chronic myocardial ischemia, sudden death, myocardial infarction, unstable or stable angina, ischemic cardiomyopathy, chronic arrhythmia, ischemic cerebrovascular disease (including stroke and multi-infarct dementia), renal hypertension, and

peripheral vascular disease, which causes intermittent claudication and gangrene. Atherosclerosis and its thrombotic complications can also cause bowel ischemia and contribute to the complications of diabetes and hypertension. Thromboembolism originating in the heart can cause embolic stroke and peripheral embolism in individuals with atrial fibrillation, acute myocardial infarction, valvular heart disease, and cardiomyopathies.

Great strides have been made in the clinical use of anticoagulants since the publication in 1984 of the first "Guide to Anticoagulant Therapy." Because of the results of well-designed randomized trials, clinicians can now make rational decisions about whether anticoagulants are indicated, the intensity of dosage regimens, the most appropriate method of laboratory monitoring, and duration of therapy.

In 1984 heparin and oral anticoagulants had established roles in the prevention and treatment of venous thromboembolism, but their roles in arterial thromboembolism were controversial. It is now clear that heparin is effective in the early treatment of unstable angina and acute myocardial infarction. The initial study in which less intense coumarin therapy was used for the treatment of venous thrombosis has now been extended to venous thrombosis prophylaxis to the prevention of systemic embolism in individuals with tissue heart valves and nonvalvular atrial fibrillation (particularly embolic stroke). Coumarins have also been shown to be effective in the long-term management of acute myocardial

infarction, but their role in this situation compared with the role of aspirin remains an open question.

HEPARIN

HISTORICAL HIGHLIGHTS

Heparin was discovered by McLean in 1916. More than 20 years later, Brinkhous and associates demonstrated that heparin requires a plasma heparin cofactor for its anticoagulant activity; this factor was renamed antithrombin III (ATIII) by Abildgaard in 1968. In the 1970s, Rosenberg *et al.* and Lindahl *et al.* elucidated the mechanisms for interactions between heparin and ATIII, demonstrating that the active center serine of thrombin and other coagulation enzymes is inhibited by an arginine reactive center on the ATIII molecule and that heparin complexes to lysine binding sites on ATIII, producing a conformational change at the arginine reactive center that converts ATIII from a slow, progressive inhibitor to a very rapid inhibitor. ATIII covalently binds to the active serine center of coagulation enzymes and heparin, then dissociates from the ternary complex and can be reutilized. It was subsequently demonstrated that heparin binds to ATIII and potentiates its activity through a unique glucosamine unit contained within a pentasaccharide sequence, the structure of which has been confirmed by chemical synthesis.

MODE OF ACTION OF HEPARIN

Only about one third of heparin binds to ATIII, and this fraction is responsible for most of its anticoagulant effect. The remaining two thirds of the heparin has minimal anticoagulant activity at therapeutic concentrations, but at high concentrations (greater than those usually produced clinically) both high- and low-affinity heparin catalyze the antithrombin effect of a second plasma protein cofactor named heparin cofactor II (HCII).

The heparin/ATIII complex inactivates a number of coagulation enzymes, including thrombin (IIa) and factors Xa, XIIa, XIa, and IXa; of these, thrombin and factor Xa are most responsive to inhibition, and human thrombin is more responsive to inhibition by the heparin/ATIII complex than factor Xa by about one order of magnitude. For inhibition of thrombin, heparin must bind to both the coagulation enzyme and ATIII, but binding to the enzyme is not required for the inhibition of activated factor X (factor Xa). Molecules of heparin with fewer than 18 saccharides are unable to bind to thrombin and ATIII simultaneously and therefore cannot catalyze thrombin inhibition. In contrast, very small heparin fragments (containing as few as six saccharides) that contain the high-affinity pentasaccharide sequence are able to catalyze the inhibition of factor Xa by ATIII. The reaction most responsive to the inhibitory effect of heparin on coagulation is the inhibition of thrombin-induced activation of factor V and factor VIII.

Heparin binds to platelets *in vitro* and can either induce or inhibit platelet aggregation, depending on experimental conditions. High molecular weight heparin fractions with low affinity for ATIII have a greater effect on platelet function than low molecular weight heparin fractions with high affinity for ATIII. Heparin prolongs bleeding time in humans and increases blood loss from the microvasculature in rabbits. The interaction of heparin with platelets and endothelial cells may contribute to heparin-induced bleeding by a mechanism independent of heparin's anticoagulant effect. Heparin also increases vessel wall permeability and suppresses the proliferation of vascular smooth muscle cells, more effects that appear to be independent of its anticoagulant activity.

Heparin is heterogeneous with respect to molecular size, anticoagulant activity, and pharmacokinetic properties. The molecular weight of heparin ranges from 3,000 to 30,000, with a mean of 15,000 (approximately 50 monosaccharide chains). The anticoagulant activity of heparin is heterogeneous because only one third of the heparin molecules administered to individuals have an anticoagulant function and because the anticoagulant profile and the clearance of heparin are influenced by the chain length of the molecules, with the higher molecular weight species being cleared from the circulation more rapidly than the lower molecular weight species. This differential clearance results in an accumulation, *in vivo*, of the lower molecular weight species, which have a reduced ratio of antithrombin to anti-factor Xa activity.

This effect is responsible for the differences observed when the relation between the heparin level and the activated partial thromboplastin time (APTT) is assessed *in vivo* and *in vitro*: the lower molecular weight species retained *in vivo* are measured in the anti-factor Xa heparin assay but have minimal effects on the APTT.

ADMINISTRATION, PHARMACOKINETICS, AND PHARMACODYNAMICS OF HEPARIN

The two preferred routes of administration of heparin are continuous intravenous infusion and subcutaneous injection. If the subcutaneous route is selected, the initial dose must be sufficiently high to compensate for the reduced bioavailability of heparin administered this way. If an immediate anticoagulant effect is required, the initial dose should be accompanied by an intravenous bolus injection because an anticoagulant effect from subcutaneous heparin is delayed for 1 to 2 hours.

After its passage into the bloodstream, heparin binds to a number of plasma proteins, a phenomenon that contributes to its reduced bioavailability at low concentrations, the variability of the anticoagulant response to fixed doses of heparin in individuals with thromboembolic disorders, and the laboratory phenomenon of heparin resistance. Binding of heparin to von Willebrand factor also results in the inhibition of von Willebrand factor-dependent platelet function. Heparin also binds to endothelial cells and macrophages, a property that

contributes to its complicated pharmacokinetics. Heparin is cleared through a combination of a rapid saturable mechanism and a much slower first-order mechanism of clearance. The mechanism of the saturable phase of heparin clearance is thought to be binding of heparin to receptors on endothelial cells and macrophages, where it is internalized and depolymerized. The slower nonsaturable mechanism of heparin clearance is largely renal. At therapeutic doses a considerable proportion of the administered heparin is cleared through the rapid, saturable, dose-dependent mechanism of clearance. Because of these kinetics, the anticoagulant effect of heparin at therapeutic doses is not linear, although both intensity and duration increase with increasing dose. Therefore the apparent biological half-life of heparin increases from approximately 30 minutes with an intravenous bolus of 25 U/kg to 60 minutes with an intravenous bolus of 100 U/kg to 150 minutes with a bolus of 400 U/kg.

The bioavailability of heparin is reduced when the drug is administered by subcutaneous injection in low doses (eg, 5,000 units every 12 hours) or moderate doses of 12,500 or even 15,000 units every 12 hours. However, at high therapeutic doses of heparin (>35,000 U per 24 hours) the plasma recovery is almost complete. The difference between the bioavailability of heparin when administered by subcutaneous or intravenous injection was strikingly demonstrated in a study of individuals with venous thrombosis (Hull *et al.* (1986) N Engl J Med 315: 1109-1114). Individuals were randomly assigned to receive either 15,000 units of heparin every 12 hours by

subcutaneous injection or 30,000 units of heparin by continuous intravenous infusion; both regimens were preceded by an intravenous bolus dose of 5,000 units. Therapeutic heparin levels and APTT ratios were achieved at 24 hours in only 37% of individuals who received subcutaneous heparin but in 71% of individuals given an identical dose of heparin by continuous intravenous infusion. These observations are relevant to the interpretation of the results of the GISSI-2 (Gruppo italiano per lo studio della sopravvivenza nell'infarto miocardico (1990) Lancet 336:65-71; Lancet (1990) 336:71-75) and ISIS-3 studies (Lancet (1992) 339: 753-770)). In these studies heparin was given in a fixed dose of 12,500 units subcutaneously twice daily beginning either 12 or 4 hours after thrombolytic therapy so that an adequate anticoagulant effect would not have been achieved in a timely manner in either study.

LABORATORY MONITORING AND DOSE-RESPONSE RELATIONS OF HEPARIN

The anticoagulant effects of heparin are usually monitored by following the results of the APTT, a test sensitive to the inhibitory effects of heparin on thrombin, factor Xa, and factor IXa. When heparin is administered in fixed doses, the anticoagulant response to it varies in individuals with acute venous thromboembolism or myocardial ischemia. Differences in the plasma concentrations of heparin-neutralizing proteins contribute to this variability. There is evidence from subgroup

analysis of cohort studies for a relation between the *ex vivo* effect of heparin on the APTT and its clinical effectiveness in the prevention of recurrent thrombosis in individuals with proximal vein thrombosis; of mural thrombosis in individuals with acute myocardial infarction; of recurrent ischemia in individuals after streptokinase therapy for acute myocardial infarction; and of coronary artery reocclusion after thrombolytic therapy with tissue plasminogen activator (TPA). Thus, in all six studies, the relative risk of an event was increased if the APTT was below the therapeutic range. For this reason the dose of heparin administered to individuals should be monitored and adjusted to achieve a therapeutic level; this anticoagulant effect is referred to as the therapeutic range.

Unfortunately, the different commercial APTT reagents vary considerably in their responsiveness to heparin. For many reagents, a therapeutic effect is achieved with an APTT ratio of 1.5 to 2.5 (measured by dividing the observed APTT by the mean of the laboratory control APTT). With very sensitive APTT reagents the therapeutic range is higher than a ratio of 1.5 to 2.5; for insensitive reagents the therapeutic range is lower. APTT reagents can be standardized by calibrating them against the heparin level (therapeutic range is 0.2 to 0.4 U/mL by protamine titration or 0.3 to 0.7 U/mL when measured using an anti-factor Xa chromogenic assay) in a plasma system.

The risk of heparin-associated bleeding increases with heparin dose (which in turn is related to the anticoagulant response), the concomitant use of thrombolytic therapy, recent surgery, trauma, invasive procedures, or a generalized hemostatic abnormality. A rapid therapeutic heparin effect is achieved by beginning with a loading dose of 5,000 units as an intravenous bolus followed by 32,000 U per 24 hours by continuous infusion. A lower dose of 24,000 U per 24 hours is often used immediately after thrombolytic therapy because the plasmolytic state produces a variable anticoagulant effect that prolongs the APTT in its own right. The APTT should be performed approximately 6 hours and 12 hours after the bolus and the heparin dose adjusted according to the result obtained. A heparin dose adjustment nomogram has been developed for APTT reagents for which the therapeutic range is 1.9 to 2.7 times control (based on a heparin level of 0.2 to 0.4 U/mL (Table 1). This nomogram is not applicable to all APTT reagents and should be adapted to the responsiveness of the local partial thromboplastin reagent to heparin.

Table 1.

Protocol for Heparin Dose Adjustment

APTT* (s)	Repeat bolus dose (U)	Stop infusion (min)	Change rate (dose) of infusion mL/h** (U per 24 h)	Time of next APTT
<50	5000	0	+3 (+2880)	6 h
50-59	0	0	+3 (+2880)	6 h
60-85	0	0	0 (0)	Next morning
86-95	0	0	-2 (-1920)	Next morning
96-120	0	30	-2 (-1920)	6 h
>120	0	60	-4 (-3840)	6 h

APTT indicates activated partial thromboplastin time; U, unit; h, hour. Starting dose of 5,000 U intravenous bolus followed by 32,000 U per 24 h as a continuous infusion, (40 U/mL). First APTT performed 6 h after the bolus injection, dosage adjustments made according to protocol and APTT repeated as indicated in the far right column.
*Normal range for APTT with Dade Actin FS reagent is 27 to 35 s.
**40 U/mL.

A therapeutic range of 60 to 85 s is equivalent to a heparin level of 0.2 to 0.4 U/mL by protamine titration or 0.35 to 0.7 U/mL as an anti-factor Xa heparin level. Therapeutic range will vary with responsiveness of APTT reagent to heparin.

It is also possible to achieve therapeutic heparin levels with subcutaneous injection in a dose of 35,000 U per 24 hours in two divided doses. The anticoagulant effects of subcutaneous heparin are delayed for approximately 1 hour and peak levels occur at approximately 3 hours.

LIMITATIONS OF HEPARIN USE

The limitations of heparin use are based on its pharmacokinetic, biophysical, and nonanticoagulant, antihemostatic properties. The pharmacokinetic limitations are due to its binding to plasma proteins and endothelial cells, which results in a complicated mechanism of clearance, as well as to heparin resistance and the variability in the anticoagulant response to fixed doses. The biophysical limitations occur because the heparin/ATIII complex is unable to access and inactivate either factor Xa in the prothrombinase complex or thrombin bound to fibrin or to subendothelial surfaces. The limitations attributable to its other (nonanticoagulant) antihemostatic properties are due to a poorly defined inhibitory effect of heparin on platelet function.

The limitations related to the pharmacokinetic and antihemostatic properties of heparin are not shared by the low molecular weight heparins and some heparinoids, and those due to the lack of accessibility of the heparin/ATIII complex to fibrin-bound thrombin and factor Xa are overcome by several new classes of ATIII-independent thrombin and factor Xa inhibitors.

The anticoagulant effect of heparin is modified by platelets, fibrin, vascular surfaces, and plasma proteins. Platelets limit the anticoagulant effect of heparin in two ways. First, factor Xa generated on the platelet surface is protected from inhibition by heparin/ATIII. Second, platelets release the heparin-neutralizing protein platelet factor IV. Fibrin binds thrombin and protects it

from inactivation by heparin/ATIII. Therefore, much higher concentrations of heparin are needed to inhibit thrombin bound to fibrin than are required to inactivate the free enzyme. Thrombin also binds to subendothelial matrix proteins, where it is again protected from inhibition by heparin. These observations explain why in experimental animals heparin is less effective than the ATIII-independent thrombin and factor Xa inhibitors at preventing thrombosis; they also raise the possibility that ATIII-independent inhibitors may be more effective than heparin in certain clinical situations.

At clinically effective doses the low molecular weight heparins and heparinoids do not have the limitations of heparin that are due to inhibition of platelet function and the associated increase in experimental microvascular bleeding; they may therefore be administered in higher doses than heparin.

CLINICAL USE OF HEPARIN

Heparin is effective in the prevention and treatment of venous thrombosis and pulmonary embolism, the prevention of mural thrombosis after myocardial infarction and of coronary artery rethrombosis after thrombolysis, and the treatment of individuals with unstable angina and acute myocardial infarction.

As noted previously, the anticoagulant response to heparin varies widely between individuals with thromboembolic disease, and the clinical efficacy of heparin is optimized if the anticoagulant effect is

maintained in a therapeutic range. For these reasons, heparin treatment is usually monitored to maintain the APTT at a level equivalent to a heparin level of 0.2 to 0.4 U/mL by protamine titration or an anti-factor Xa level of 0.35 to 0.7 U/mL. For many APTT reagents this is equivalent to a ratio (test APTT/lab control APTT) of 1.5 to 2.5, the therapeutic range. This recommended therapeutic range is supported by evidence from animal studies and by subgroup analysis of prospective cohort studies of the treatment of deep vein thrombosis, of the prevention of mural thrombosis after myocardial infarction, and the prevention of recurrent ischemia after coronary thrombolysis.

TREATMENT OF VENOUS THROMBOEMBOLISM

Evidence for the effectiveness of heparin in the treatment of pulmonary embolism comes from the randomized study by Barritt and Jordon (Barritt and Jordon (1960) Lancet 1: 1309-1312), who reported an impressive reduction in mortality in individuals receiving heparin plus oral anticoagulants compared with individuals in an untreated control group. Heparin was effective in the treatment of venous thrombosis in a randomized study that demonstrated that both symptomatic and asymptomatic recurrences as well as complicating pulmonary embolism were much more common in individuals with acute proximal vein thrombosis who received oral anticoagulants without concomitant heparin than in those receiving oral anticoagulants and heparin. In addition, in two randomized studies recurrent

thrombosis was very uncommon (less than 5%) during the initial course of intravenous heparin but, was common (between 29% to 47%) if full-dose heparin was discontinued after 5 to 14 days. Recurrence is markedly reduced if the initial course of heparin is followed by oral anticoagulants or adjusted-dose heparin.

Heparin administered by continuous intravenous infusion has been compared in terms of effectiveness and safety with heparin administered by intermittent intravenous injection in six studies. The continuous intravenous heparin infusion route has also been compared with high-dose subcutaneous heparin in six other studies. However, it is difficult to identify the optimal route of heparin administration from the results of these studies for several reasons: 1) different 24-hour heparin doses were used; 2) most of the studies were small and lacked the statistical power to demonstrate clinically important differences; and 3) different criteria were used to assess both efficacy and safety. Nevertheless, the results of these studies indicate that the risk of bleeding increases with heparin dose; both the continuous intravenous route and the subcutaneous route are safe and effective; and the frequency of recurrent venous thromboembolism is low with all three methods of administration, provided that adequate doses of heparin are given.

In all the contemporary studies in which objective tests were used to assess outcomes, the mean daily dose of heparin has been between 30,000 and 35,000 U per 24 hours. The initial dose of heparin is critical, especially if

heparin is administered by subcutaneous injection, because an adequate anticoagulant response is not achieved in the first 24 hours unless a high starting dose (17,500 units subcutaneously as the initial injection) is used. The most reliable estimates of the incidence of recurrence during adequate heparin treatment and over the subsequent 3 months of less intense warfarin therapy come from three contemporary prospective studies of a total of 523 individuals to whom heparin was administered by continuous infusion. The dose of heparin was adjusted to maintain the APTT in the therapeutic range, follow-up was prospective, and diagnosis of recurrence was based on reliable objective tests. The 3-month incidence of recurrent venous thromboembolism varied from 4.7% to 7.1% over the combined period of initial heparin treatment and subsequent oral anticoagulant therapy. The incidence of major bleeding during heparin treatment varied from 1.6% to 7.1% (mean, 3.8%) and the incidence of fatal pulmonary embolism was 0%(Table 2).

Table 2

Confirmed Recurrences, Major Bleeding Events, and Fatal Pulmonary Embolism Among Individuals With Venous Thromboembolism Who Receive Treatment With Heparin Administered as a Continuous Intravenous Infusion and Followed by Administration of Less Intense Oral Anticoagulants

Study	Incidence of confirmed recurrence*	Incidence of major bleeding**	Incidence of fatal pulmonary embolism*
Gallus *et al* (1986) Lancet 2:1293-1296	13 /266 (4.9%)	5 /266 (1.9%)	. . .
Hull *et al* (1986) N. Engl J Med 315: 1109-1114	3 /58 (5.2%)	2 /58 (3.4%)	0 /58 (0%)
Hull *et al* (1990) N Engl J Med 322:1260-1264	14 /199 (7.0%)	13 /199 (6.5%)	0 /199 (0%)
Total	30 /523 (5.7%)	20 /523 (3.8%)	0 /257

*During the 3-mo period of heparin and oral anticoagulant treatment.
**During heparin treatment.

Audits of heparin monitoring practices indicate that the dosage adjustments are frequently inadequate. Dosing practices can be improved by using a standardized approach. In a prospective study, heparin was given intravenously as a continuous infusion, starting at a dose of approximately 31,000 U per 24 hours after a 5,000-unit intravenous bolus, and the dose was then adjusted using a heparin protocol developed empirically through an iterative process (Table 1). An APTT above the lower limit of the therapeutic range was reached in 82% of

individuals at 24 hours and in 91% at 48 hours. The mean heparin dose required to produce an APTT in the therapeutic range was 32,903 U per 24 hours. The proportion of APTT results in the therapeutic range was significantly higher when the heparin protocol was used than in a historical control group ($P < 0.05$). It should be noted, however, that the protocol was developed for a single APTT reagent (Dade Actin™) and should be modified for other reagents.

The time-honored approach of using a 7- to 10-day course of heparin with a 4- to 5-day overlap period with oral anticoagulants has been challenged by the results of two randomized studies in individuals with proximal vein thrombosis. In these studies the low recurrence rate and bleeding incidence with a short course of heparin therapy (4 to 5 days) were similar to those with a longer course (9 to 10 days). The short-course regimen has obvious appeal; it reduces hospital stay and lessens the risk of heparin-associated thrombocytopenia. Although the shorter course of treatment can be recommended for the average individual with venous thromboembolism, it may not be appropriate for individuals with massive iliofemoral vein thrombosis or major pulmonary embolism, because these individuals were excluded from one study and constituted only a small proportion of individuals in the second.

PROPHYLAXIS OF VENOUS THROMBOEMBOLISM

Heparin in a fixed low dose of 5,000 units subcutaneously every 8 or 12 hours is an effective and

safe form of prophylaxis in medical and surgical individuals at risk for venous thromboembolism. Overview analyses of clinical trials in individuals undergoing elective general surgery and in medical individuals have reported that low-dose heparin produces a 60% to 70% risk reduction in venous thrombosis and in fatal pulmonary embolism. In one analysis the incidence of fatal pulmonary embolism was 0.7% in the control group and 0.2% in treated general surgical individuals ($P<0.001$); in another, in which orthopedic surgical individuals were included, the results were 0.8% and 0.26% respectively ($P<0.001$), and there was also a small but statistically significant difference in mortality (3.3% and 2.4%, respectively; $P<0.02$). The use of low-dose heparin is associated with a small excess of wound hematoma and a minimal, nonsignificant increase in major bleeding but no increase in fatal bleeding. Low-dose heparin has also been shown to be effective in reducing venous thromboembolism after myocardial infarction and in individuals with other serious medical disorders and to reduce in-hospital mortality by 31% ($P<0.05$) among 1358 individuals over the age of 40 who were admitted to general medical wards. Low-dose heparin is also effective in reducing deep-vein thrombosis after hip surgery. The risk of thrombosis, however, remains substantial at an incidence of 20% to 30% and can be reduced further by treatment with either adjusted low-dose heparin or fixed-dose low molecular weight heparin. Moderate-dose warfarin is effective in individuals undergoing major orthopedic surgical procedures, but direct comparisons of low-dose

heparin and warfarin have not been performed in these individuals.

CORONARY ARTERY DISEASE

Coronary thrombosis is important in the pathogenesis of several acute complications of coronary artery disease: unstable angina and its complications, acute myocardial infarction, and many cases of sudden deaths; and recurrent infarction and death in individuals with acute myocardial infarction who are treated with thrombolytic therapy. Heparin has the potential to prevent the acute thrombotic manifestations of coronary artery disease, but its clinical use cannot be considered in isolation; rather, it must be considered when combined with standard treatment, which is aspirin in all potentially eligible individuals with myocardial ischemia and both aspirin and thrombolytic therapy in individuals with evolving myocardial infarction. Unfortunately, studies using clinically important outcomes to evaluate the benefits and risks of adding heparin to aspirin alone or to aspirin and thrombolytic therapy are relatively few, and the results have been inconclusive.

UNSTABLE ANGINA

Four large trials (Lewis *et al.* (1983) N Engl J Med 309: 396-403; Cairns *et al.* (1985) N Engl J Med 313: 1369-1375; Théroux *et al.* (1988) N Engl J Med 319: 1105-1111; Lancet (1990) 336: 827-830) in which aspirin was

given to individuals with unstable angina have shown marked reductions of acute myocardial infarction and cardiac death in both the short and long term. The suggestion has been made that the addition of heparin to aspirin improves short-term outcome. Heparin when used alone is also effective in the short term in preventing acute myocardial infarction and recurrent refractory angina in individuals with unstable angina, but a rebound is seen when heparin is stopped. Aspirin appears to prevent the cluster of ischemic events that occur when heparin is discontinued.

ACUTE MYOCARDIAL INFARCTION

Heparin reduced reinfarction and death in two open randomized trials in which a heparin group was compared with an untreated control group. In one study, there was a statistically significant 61% reduction in reinfarction when 12,500 units of heparin was given subcutaneously to individuals who had had a myocardial infarction 6 to 18 months before recruitment into the study (Neri Serneri *et al.* (1987) Lancet 1: 937-942). In another study there was a significant 44% reduction in mortality when 12,500 units of heparin was given subcutaneously every 12 hours to individuals with acute myocardial infarction (SCATI Group (1989) Lancet 2:182-186). In neither of these studies were the added benefits or risks of adding heparin to aspirin evaluated. Therefore, the results of these studies may not be relevant to the current situation in which

individuals with acute or previous myocardial infarction are treated with aspirin.

The effect of heparin on the incidence of mural thrombosis was evaluated in two randomized trials (Turpie *et al.* (1989) N Engl J Med 320:352-357; SCATI Group (1989) Lancet 2:182-186)) in which individuals taking moderate-dose heparin (12,500 units subcutaneously every 12 hours)(13) were compared with either an untreated control group or individuals taking low-dose heparin (5,000 units subcutaneously every 12 hours) (Turpie *et al.* (1989) N Engl J Med 320:352-357). In these two studies, the incidence of mural thrombosis detected by two-dimensional echocardiography was 72% and 58% lower, respectively ($P<0.05$), in the individuals taking moderate-dose heparin than in the comparison groups.

The effectiveness of heparin in preventing early coronary artery reocclusion after successful thrombolysis has been evaluated in a number of studies. In one study, a single intravenous bolus of 10,000 units did not appear to influence coronary artery patency at 90 minutes (Topol *et al.* (1989) Circulation 79:281-286). In four other studies in which TPA was used (Hsia *et al.* (1990) N Engl J Med 323:1433-1437; Bleich *et al.* (1990) Am J Cardiol 66:1412-1417; de Bono *et al.* (1992) Br Heart J 67:122-128; National Heart Foundation of Australia Coronary Thrombolysis Group (1989) Circulation 80(suppl II):II-114, Abstract) heparin was administered as an intravenous bolus of 5,000 units and then as a continuous infusion of 1,000 U/h either during or at the end of a TPA infusion. The

dose of heparin was adjusted to maintain the APTT at 1.5 to 2.0 times control. In the Heparin-Aspirin Reperfusion Trial (Hsia *et al.* (1990) N Engl J Med 323:1433-1437) of 205 individuals (the comparison group received 80 mg of aspirin per day. Coronary artery patency at 18 hours was 82% in the heparin group and 52% in the aspirin group ($P<0.0002$). The conclusion that heparin is more effective than aspirin in maintaining patency has been criticized because the aspirin dose was too low to have a rapid and marked suppressive effect on thromboxane A_2 production. In the trial reported by Bleich and associates (Bleich *et al.* (1990) Am J Cardiol 66:1412-1417) of 83 individuals, the control group received no treatment. Patency at 2 days was 71% in the heparin group and 44% in the control group ($P<0.023$). In the European Coronary Study Group-6 Trial (de Bono *et al.* (1992) Br Heart J 67:122-128), all 687 individuals received aspirin and were randomly assigned to receive either heparin or no heparin. Patency at 81 hours was 80% in the heparin group and 75% in the control group ($P<0.01$). In the Australian National Heart Study Trial (National Heart Foundation of Australia Coronary Thrombolysis Group (1989) Circulation 80(suppl II):II-114, Abstract), all 202 individuals received heparin for 24 hours. They were then randomly assigned to receive either continuous intravenous heparin or a combination of aspirin (300 mg) and dipyridamole (300 mg) daily. Patency at 1 week was 80% in both groups. The results of these studies suggest that heparin in a dose of 5,000 units by intravenous bolus and 1,000 U/h by continuous infusion increases patency during the first few days after coronary

thrombolysis with TPA, probably by preventing rethrombosis.

In two other studies the effect of adding heparin to aspirin given in adequate doses has been evaluated. The OSIRIS investigators treated 128 individuals with streptokinase and aspirin and randomly assigned the individuals to receive either an intravenous bolus of heparin or no heparin. There was no difference in coronary patency at 24 hours (86% and 87%) (Col *et al.* (1992) Circulation 86(suppl 1):I-259, Abstract). The Duke University Clinical Cardiology Studies-1 investigators (O'Connor *et al.* (1994) J Am Coll Cardiol 23:11-18) treated 250 individuals with anisoylated plasminogen-streptokinase activator complex and aspirin and randomly assigned the individuals to receive either heparin or no heparin. There was no significant difference in coronary artery patency (80% in the heparin group and 74% in the control group).

Subgroup analysis of the European Coronary Study Group-6 Trial (de Bono *et al.* (1992) Br Heart J 67:122-128) and the Heparin-Aspirin Reperfusion Trial (Hsia *et al.* (1990) N Engl J Med 323:1433-1437) revealed some interesting and provocative results. In both studies, heparin was given intravenously in a fixed dose, and the APTT was performed but was not used to adjust the dose of heparin in a systematic manner. In the Heparin-Aspirin Reperfusion Trial, the subgroup of individuals whose APTT ratio was considered optimal had a significantly higher patency rate than those whose APTT ratio was suboptimal:

patency was 45% in those whose APTT was <45 seconds; 88% in those whose APTT was >45 seconds but <60 seconds; and 95% in those whose APTT was >60 seconds. These findings suggest that the effectiveness of heparin in maintaining patency is dependent on keeping APTT in the therapeutic range, and that coronary patency achieved with TPA is improved by using high-dose intravenous heparin in therapeutic doses.

The effectiveness of heparin in preventing reinfarction or death after thrombolytic therapy for acute myocardial infarction has been evaluated in a number of randomized studies. In the ISIS-2 study (ISIS-2 Collaborative Group (1988) Lancet 2:349-360), approximately half of the individuals received intravenous heparin over 48 hours in a 2x2 factorial design that included streptokinase and aspirin; heparin treatment was associated with a nonsignificant decrease in recurrent infarction. In the Studio Sulla Calciparina Nell'Angina e Nella Trombosi Ventricolare Nell'Infarto study (SCATI Group (1989) Lancet 2:182-186), in which the control group received no anticoagulant treatment, mortality was reduced significantly in individuals randomly assigned to receive heparin (2,000 units intravenous bolus followed by 12,500 units administered subcutaneously every 12 hours) after thrombolytic therapy for acute myocardial infarction on a subgroup analysis. The same trend was seen with streptokinase but not with TPA in the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI-2)/International Study. In the individuals who received streptokinase and heparin (90% of whom also

received aspirin) the mortality rate was 7.9% (408/5191); in the individuals who received streptokinase alone it was 9.2% (479/5205) (P<0.02). When individuals who died before heparin was started were excluded from the analysis, the same trend was still apparent; the rates were 5.0% (254/5037) and 6.2% (311/5037) (P<0.02). The mortality rate among the individuals who received TPA and heparin was 9.2% (476/5170), and was 8.7% (453/5202) in those who received TPA not followed by heparin (P=0.393). After the individuals who died before heparin was started were excluded from analysis, the mortality rate, 5.9%, was identical for those who received heparin (298/5047) and those who did not (298/5047) (P=0.984).

The ISIS-3 study provides additional important information on the relative safety and efficacy of adjuvant heparin and on the relative safety of streptokinase and TPA (ISIS-3 (1992) Lancet 339:753-770). The addition of heparin (12,500 units subcutaneously every 12 hours, starting 4 hours after thrombolytic therapy was begun) to aspirin and thrombolytic therapy produced a small excess of major noncerebral bleeds (1.0% compared with 0.8%; P<0.01) and of cerebral bleeds (0.56% compared with 0.40%; P<0.05). Thus, the addition of heparin resulted in an excess of 3.6 events per 1000 serious bleeding events. On the other hand, the addition of heparin resulted in a reduction of reinfarction of 3.1 events per 1000 treated (P<0.09) and a reduction in 35-day mortality of 3 events per 1000 treated (difference not significant). The incidences of stroke and of stroke from presumed cerebral hemorrhage were significantly lower in

individuals receiving streptokinase than in those given TPA or anisoylated plasminogen-streptokinase activator complex. Thus, compared with streptokinase, TPA was associated with an excess of 3.5 strokes per 1000 and 4.2 episodes of presumed hemorrhagic strokes per 1000 (stroke rate, 1.04% for streptokinase and 1.39% for TPA; cerebral hemorrhage rate, 0.24% for streptokinase and 0.66% for TPA; $P<0.05$ for both comparisons). Based on these findings, it seems possible that any additional benefit from higher-dose and monitored intravenous heparin will be associated with an increase in hemorrhagic stroke.

However, the results of the recently completed Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries study (GUSTO Angiographic Investigators (1993) N Engl J Med 329:1615-1622) indicate otherwise. In this multinational study, 41,021 individuals with evolving myocardial infarction were randomly assigned to treatment with four different strategies: 1) streptokinase and subcutaneous heparin, 2) streptokinase and intravenous heparin, 3) accelerated TPA and intravenous heparin, or 4) the combination of both thrombolytic agents with intravenous heparin. The mortality for the four treatment groups was 7.2%, 7.4%, 6.3%, and 7.0%, respectively. The 14% reduction of mortality in the group receiving TPA, compared with the mortality in the groups receiving the streptokinase strategies, was highly significant ($P=0.001$). The rates of hemorrhagic stroke were 0.49%, 0.54%, 0.72%, and 0.94%, respectively, reflecting a significant excess of events in the TPA group compared with the streptokinase group

(P=0.03). The combined incidence of death or nonfatal hemorrhagic stroke was significantly reduced for the TPA group compared with the groups receiving streptokinase (6.6% and 7.5%, respectively; P=0.004). There was no difference between the TPA and streptokinase groups in terms of extracranial bleeding. The incidence of severe or life-threatening bleeding was 0.3%, 0.5%, and 0.4%, respectively, in the groups receiving streptokinase and subcutaneous heparin, streptokinase and intravenous heparin, and TPA and intravenous heparin. The incidence of moderate or severe bleeding was 5.8%, 6.3%, and 5.4%, respectively. Thus, the improved survival seen in the group receiving TPA and high-dose intravenous heparin was associated with a small increase in the risk of hemorrhagic stroke and no increase in major extracranial bleeding. In contrast, there was no advantage to using intravenous heparin in individuals treated with streptokinase.

BLEEDING

The results of the GISSI-2 and the ISIS-3 studies show that the addition of heparin therapy to thrombolytic treatment increases the risk of bleeding (GISSI-2 (1990) Lancet 336:65-71; Lancet (1990) 336:71-75; ISIS-3 (1992) Lancet 339:753-770): minor bleeds were reported for 594 of the 6,195 individuals (9.6%) who received heparin and 328 of the 6,206 (5.3%) who did not (relative risk, 1.88; P<0.001; GISSI centers only), and major bleeds were reported for 103 of 10,361 individuals (1%) in the heparin

group and 57 of 10,407 (0.5%) in the group who did not receive heparin (relative risk, 1.79; P<0.01). As discussed above, in the ISIS-3 study heparin produced a small but significant excess of major bleeding episodes and cerebral hemorrhage. In the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries study (GUSTO Angiographic Investigators (1993) N Engl J Med 329:1615-1622) there was a small but significant increase in the incidence of cerebral hemorrhage in the group receiving accelerated TPA and intravenous heparin, no difference in the incidence of hemorrhagic stroke between the intravenous and subcutaneous arms of the streptokinase groups, and no difference in the incidence of major extracranial bleeding between these three groups.

LOW MOLECULAR WEIGHT HEPARINS AND HEPARINOIDS

The experimental observations with low molecular weight heparins of the 1970s and early 1980s led to clinical trials that demonstrated the effectiveness and safety of these antithrombotic agents for the prevention and treatment of venous thrombosis.

Low molecular weight heparins are approximately one third the size of heparin. Like heparin, they are heterogeneous in size with a molecular weight range of 1,000 to 10,000 and a mean molecular weight of 4,000 to 5,000. Depolymerization of heparin results in a change in its anticoagulant profile, bioavailability and pharmacokinetics, and effects on platelet function and

experimental bleeding. The Organon heparinoid Orgaran™, a mixture of 80% heparan sulfate and smaller amounts of dermatan sulfate and chondroitin sulfates, has also been tested clinically.

ANTICOAGULANT EFFECTS OF LOW MOLECULAR WEIGHT HEPARINS

Like heparin, low molecular weight heparins produce their major anticoagulant effect by binding to ATIII through a unique pentasaccharide sequence. This sequence, all that is necessary for factor Xa inhibition, is present on less than one third of low-molecular weight heparin molecules. A minimum chain length of 18 saccharides (including the pentasaccharide sequence) is required for thrombin inhibition. Virtually all the heparin molecules of standard heparin contain at least 18 saccharide units, whereas only 25% to 50% of the different low-molecular weight heparins contain fragments with 18 or more. Therefore, compared with unfractionated heparin, which has a ratio of anti-factor Xa to anti-factor IIa activity of approximately 1:1, the various commercial low-molecular weight heparins have anti-factor Xa to anti-IIa ratios that vary between 4:1 and 2:1, depending on their molecular size distribution.

PHARMACOKINETICS OF LOW MOLECULAR WEIGHT HEPARINS

The bioavailability and pharmacokinetics of low molecular weight heparins differ from those of heparin

because of differences in the binding properties of the two sulfated polysaccharides.

Low molecular weight heparins bind much less avidly to heparin-binding proteins than does heparin, a property that contributes to their superior bioavailability at low doses and their more predictable anticoagulant response. They also do not bind to endothelial cells in culture, a property that could account for their longer plasma half-life. Low molecular weight heparins are cleared principally by the renal route, and their biological half-life is increased in individuals with renal failure. Preparations of low molecular weight heparins also have a lower affinity than heparin for von Willebrand factor, in accord with the observation that they produce less experimental bleeding than heparin for equivalent antithrombotic effects.

ANTITHROMBOTIC AND HEMORRHAGIC EFFECTS IN ANIMAL MODELS

The antithrombotic and hemorrhagic effects of heparin have been compared with those of low molecular weight heparins, the Orgaran™ heparinoid, and dermatan sulfate in a variety of animal models. In these models of thrombosis, temporary venous stasis is produced by ligating an appropriate vein, and blood coagulation is stimulated by injection of serum, factor Xa, thrombin, or tissue factor. When compared on a gravimetric basis, low molecular weight heparins are slightly less effective antithrombotic agents than heparin but produce much less bleeding in models used to measure blood loss from a

standardized injury. These differences in the relative antithrombotic to hemorrhagic ratios among these sulfated polysaccharides could be due in part to their different effects on platelet function and vascular permeability.

REVERSAL OF ANTICOAGULANT AND HEMORRHAGIC EFFECTS WITH PROTAMINE

Protamine sulfate neutralizes the anticoagulant effect of heparin, but it does not completely neutralize the anticoagulant activity of low molecular weight heparins. It is likely that protamine forms complexes with the higher molecular weight fractions of these heparins but not with the very low molecular weight fractions. Nevertheless, in an experimental model of microvascular bleeding in rabbits, protamine sulfate completely neutralized abnormal blood loss induced by both heparin and a low molecular weight heparin, even though it only partly neutralized the anti-factor Xa activity ex vivo.

CLINICAL STUDIES

Low molecular weight heparins have a longer plasma half-life and a more predictable anticoagulant response than unfractionated heparin, so they can be administered once daily and without laboratory monitoring. In animal models they produce less bleeding than heparin for an equivalent antithrombotic effect, so individuals can be treated with doses of low molecular weight heparins that

produce a higher anti-factor Xa level than unfractionated heparin without safety being compromised. This potential advantage of low molecular weight heparins has been demonstrated in one study of prophylaxis in which heparin produced a significant increase in bleeding when the dose was increased to match the anticoagulant effect *ex vivo* of a low molecular weight heparin (Levine *et al.* (1991) Ann Intern Med 114:545-551), as well as in two studies in which high doses of a low molecular weight heparin were compared with full doses of heparin for the treatment of venous thrombosis (Hull *et al.* (1992) N Engl J Med 326:975-982; Prandoni *et al.* (1992) Lancet 339:441-445). Low molecular weight heparins have been evaluated for the prevention and treatment of venous thromboembolism and are highly effective.

PREVENTION OF VENOUS THROMBOSIS

GENERAL SURGERY

Low molecular weight heparins were found to be effective and safe in two well-designed randomized trials in which a group treated with them was compared with an untreated control group (Ockelford *et al.* (1989) Thromb Haemost 62:1046-1049; Pezzuoli *et al.* (1989) Int Surg 74:205-210). In one study, there was an increase in minor bleeding in the group taking them (compared with the group taking placebo)(Pezzuoli *et al.* (1989) Int Surg 74:205-210) but in neither was there an increased incidence of major hemorrhage (Ockelford *et al.* (1989) Thromb Haemost 62:1046-1049; Pezzuoli *et al.* (1989) Int Surg 74:205-210).

In one study of 4,498 individuals there was a statistically significant reduction in thromboembolic mortality in those taking low molecular weight heparins (0.36% compared with 0.09% [risk reduction, 75%]) (Pezzuoli *et al.* (1989) Int Surg 74:205-210). In the other a marked risk reduction in fibrinogen scan-detected thrombi was observed (Ockelford *et al.* (1989) Thromb Haemost 62:1046-1049).

In two studies low molecular weight heparin was more effective than low-dose heparin (EFS Group (1988) Br J Surg 75:1058-1063; Kakkar and Murray (1985) Br J Surg 72:786-791), but in six other studies (Bergqvist *et al.* (1988) Br J Surg 75:888-891; Caen (1988) Thromb Haemost 59:216-220; Hartl *et al.* (1990) Thromb Res 57:577-584; Fricker *et al.* (1988) Eur J Clin Invest 18:561-567; Liezorovicz *et al.* (1991) Br J Surg 78:412-416; Samama *et al.* (1988) Br J Surg 75:128-131) there was no significant difference in efficacy. In six of these eight studies there was no difference in the incidence of bleeding; in one study bleeding was significantly less in the low molecular weight heparin group (Fricker *et al.* (1988) Eur J Clin Invest 18:561-567); and in another, bleeding was significantly greater in that group (Bergqvist *et al.* (1988) Br J Surg 75:888-891).

ORTHOPEDIC SURGERY

Compared with placebo, treatment with low molecular weight heparins resulted in a risk reduction for all thrombi and for proximal vein thrombi of between 70%

and 79%. This reduction occurred without an increase in clinically important bleeding in two studies (Leclerc *et al.* (1992) Thromb Haemost 67:417-423; Turpie *et al.* (1986) N Engl J Med 315:925-929) and with a small increase in minor bleeding in a third (Hoek *et al.* (1989) Thromb Haemost 67:28-32).

The use of low molecular weight heparins has been compared with a variety of other methods of prophylaxis during orthopedic surgery, including low-dose heparin, low-dose heparin and dihydroergotamine, adjusted-dose heparin, Dextran, and warfarin.

Low molecular weight heparins were significantly more effective than standard low-dose heparin, and there was a trend for a decrease in major bleeding.

In the limited number of comparative trials, low molecular weight heparins were as effective and safe as adjusted-dose heparin (Leyvraz *et al.* (1991) Br Med J 303:543-548; Dechavanne *et al.* (1989) Haemostasis 19:5-12) and warfarin (Heit *et al.* (1991) Blood 778:187A, Abstract) in individuals having elective hip surgery and were much more effective than Dextran. They were also more effective than warfarin in individuals having major knee surgery.

There has only been one randomized trial evaluating a low molecular weight heparin or heparinoid in individuals with hip fracture (Heit *et al.* (1991) Blood 778:187A, Abstract). In this trial, Orgaran™ was compared to Dextran, both drug regimens beginning preoperatively. The incidence of thrombosis was 10% in the Orgaran group

and 30% in the Dextran group ($P<0.001$). However, the number of units of blood transfused was significantly higher in the group given Dextran.

MEDICAL INDIVIDUALS

Low molecular weight heparins are very effective and safe prophylactic agents in medical individuals and have been compared with placebo in two studies of individuals with ischemic stroke (Prins *et al.* (1987) Thromb Haemost 58(suppl):117, Abstract; Turpie *et al.* (1987) Lancet 1:523-526) and in one study of high-risk medical individuals over the age of 65 years (Dahan *et al.* (1986) Haemostasis 16:159-164). Low molecular weight heparins have also been compared with low-dose heparin in two studies (Green *et al.* (1990) Ann Intern Med 113:571-574; Turpie *et al.* (1992) Ann Intern Med 117:353-357). In all of the reported studies, fibrinogen leg scanning was used to detect venous thrombosis. Compared with placebo, treatment with low molecular weight heparins produced a relative risk reduction in venous thrombosis of between 40% and 86% in individuals with stroke and in high-risk medical individuals; this effect was seen without an increase in clinically important bleeding. In both studies in which low molecular weight heparins were compared with heparin, individuals randomly assigned to receive low molecular weight heparin had a statistically significant relative risk reduction for thrombosis of greater than 70% (Green *et al.* (1990) Ann Intern Med

113:571-574; Turpie *et al.* (1992) Ann Intern Med 117:353-357).

TREATMENT OF ESTABLISHED THROMBOSIS

Low molecular weight heparins have been compared with standard heparin in six relatively large studies (Hull *et al.* (1992) N Engl J Med 326:975-982; Prandoni *et al.* (1992) Lancet 339:441-445; Albada *et al.* (1989) Circulation 80:935-940; Bratt *et al.* (1990) Thromb Haemost 64:506-510; Thromb Haemost (1991) 65:251-256; Simonneau *et al.* (1993) Arch Intern Med 153:1541-1546). Most of the randomized trials used a change in thrombus size between the pretreatment and 5- to 10-day posttreatment venograms as the outcome measure. In all studies, low molecular weight heparins were at least as effective as unfractionated heparin in preventing extension of venous thrombosis, and in most they were associated with a greater reduction in thrombus size than heparin. In most of these studies, unfractionated heparin was administered by continuous intravenous infusion and was monitored to maintain the APTT in a defined therapeutic range, and the low molecular weight heparin was usually administered by subcutaneous injection without laboratory monitoring.

Two recent large studies (Hull *et al.* (1992) N Engl J Med 326:975-982; Prandoni *et al.* (1992) Lancet 339:441-445) used the more clinically relevant end point of confirmed symptomatic recurrent thromboembolism as the outcome measure. The results of these studies indicate that in individuals with proximal vein thrombosis, low

molecular weight heparins administered by subcutaneous injection in a fixed dose or weight-adjusted dose are at least as safe and probably more effective than conventional standard heparin administered by continuous infusion and monitored with the APTT.

RECOMMENDATIONS

Firm recommendations based on solid evidence can be made for the prevention and treatment of venous thromboembolism and for the treatment of unstable angina. The evidence supporting specific dosage regimens for the treatment of acute myocardial infarction is less conclusive and subject to revision. In all cases of treatment, the dose of heparin should be adjusted to maintain the APTT at a ratio of 1.5 to 2.5 times control (equivalent to a heparin level of 0.2 to 0.4 U/mL by protamine titration).

TREATMENT OF VENOUS THROMBOEMBOLISM

Individuals with venous thromboembolism should be treated with a 5,000-unit intravenous bolus of heparin followed by either 32,000 U per 24 hours by continuous infusion or 17,500 U subcutaneously every 12 hours, and the dose should be adjusted to maintain the APTT at 6 hours within the therapeutic range of 1.5 to 2.5 times control.

PREVENTION OF VENOUS THROMBOEMBOLISM

General surgical and medical individuals should receive 5,000 U of heparin subcutaneously every 12 hours. Individuals having major orthopedic surgery or very high-risk individuals (those with a history of recurrent venous thrombosis) should receive low molecular weight heparin, adjusted low-dose heparin (adjusted to the upper normal APTT range), or less intense warfarin. Of these regimens, low molecular weight heparin has the advantage of being more convenient because it does not require monitoring. It is also more effective than warfarin in individuals undergoing major knee surgery. In addition, low molecular weight heparin is more effective than adjusted-dose standard heparin in reducing the incidence of proximal deep vein thrombosis after elective hip surgery.

TREATMENT OF UNSTABLE ANGINA OR ACUTE MYOCARDIAL INFARCTION

If thrombolytic therapy is not given, individuals with unstable angina or acute myocardial infarction should receive 325 mg aspirin and 5,000 U heparin as an intravenous bolus followed by 32,000 U per 24 hours. If thrombolytic therapy is used, the need for added heparin therapy is less clear. If a decision is made to use heparin, it should be given in a dose of 24,000 U per 24 hours. Heparin should be given concomitantly with TPA but can be delayed for 2 to 3 hours after streptokinase.

SIDE EFFECTS OF HEPARIN

The most common side effect of heparin is hemorrhage. Other complications are thrombocytopenia with or without thrombosis, osteoporosis, skin necrosis, alopecia, hypersensitivity reactions, and hypoaldosteronism. Four variables have been reported to influence bleeding during heparin treatment: the dose of heparin, the individual's anticoagulant response, the method of heparin administration, and individual factors. There is indirect evidence that the frequency of bleeding is increased by heparin dose and anticoagulant effect. Pooled analysis of randomized trials in which different methods of heparin administration were compared shows an average incidence of major bleeding of 6.8% in the continuous infusion group and 14.2% in the intermittent intravenous group (odds ratio, 0.42; P=0.01). However, this comparison is confounded by the difference in the 24-hour heparin dose, which was greater in the intermittent intravenous group in five of the six studies; the observed increase in bleeding could have been contributed to by the higher dose of heparin. For studies in which continuous intravenous heparin was compared with subcutaneous heparin, the average incidence of bleeding was 4.4% and 4.3%, respectively (odds ratio, 1.0). Other factors that predispose the individual to anticoagulant-induced bleeding are serious concurrent illnesses and chronic heavy alcohol consumption.

The concomitant use of aspirin has long been identified as a risk factor for heparin-induced bleeding.

Aspirin increases operative and postoperative bleeding in individuals who receive the very high doses of heparin required during open-heart surgery. However, the risk of adding aspirin to a short course of regular therapeutic doses of heparin is likely to be much lower, and is acceptable in individuals with ischemic heart disease.

Renal failure and individual age and gender have also been implicated as risk factors for heparin-induced bleeding. The reported association with female gender is not consistent and remains in question.

Thrombocytopenia, a well-recognized complication of heparin therapy, has been reviewed recently. In most cases, it is asymptomatic. The reported incidence of heparin-associated thrombocytopenia varies widely. Thrombocytopenia is more common with heparin derived from bovine lung than with that from porcine gut. Pooled ánalysis of studies in which individuals were randomly assigned to receive heparin derived from different sources revealed an overall incidence of thrombocytopenia of 15.6% in the 173 individuals receiving bovine heparin and 5.8% in the 223 individuals receiving porcine heparin (King and Kelton (1984) Ann Intern Med 100:535-540). On pooled analysis of all prospective studies with porcine heparin, the mean incidence of thrombocytopenia is 2.4% for therapeutic heparin and 0.3% for prophylactic heparin. The incidence of arterial or venous thrombosis with heparin-associated thrombocytopenia is approximately 0.4%. Arterial thrombosis occurs as a consequence of platelet aggregation *in vivo*, but venous thrombosis could result

from heparin resistance caused by the neutralizing effect of heparin-induced release of platelet factor IV. Thrombocytopenia usually begins between 3 and 15 days (median, 10 days) after heparin therapy is started, but it has been reported within hours of the start of heparin therapy in individuals previously exposed to heparin. The platelet count usually returns to baseline levels within 4 days after heparin is stopped. Heparin-associated thrombocytopenia is thought to be caused by an IgG-heparin immune complex involving both the Fab and Fc portion of the IgG molecule. Although low molecular weight heparins can exhibit immunologic cross-reactivity with heparin, the heparinoid Orgaran™ exhibits minimal cross-reactivity and has been used successfully to manage a small number of individuals with heparin-associated thrombocytopenia.

ORAL ANTICOAGULANTS

The oral anticoagulants, or vitamin K antagonists, are coumarins or indandione derivatives. Coumarins produce fewer nonhemorrhagic complications and are now used exclusively, and of these, warfarin (4-hydroxycoumarin) is the most widely used in the United States. Since publication of the first "Guide to Anticoagulant Therapy" in 1984, the mode of action of coumarins has been clarified; their role in the management of a number of thromboembolic disorders has been more clearly defined by evaluation in randomized clinical trials; a system has been introduced to standardize the measurement and reporting of their anticoagulant effect; and the safety of

their use for number of indications has been improved, without compromising efficacy, by the adoption of a less intense target therapeutic range than was previously used in the United States.

PHARMACOLOGY

MECHANISM OF ACTION

Oral anticoagulants produce their effect by interfering with the cyclic interconversion of vitamin K and its 2,3 epoxide (vitamin K epoxide) (Fig. 10). Vitamin K is a necessary cofactor for the posttranslational carboxylation of glutamate residues to -carboxyglutamates on the N-terminal regions of vitamin K-dependent proteins. -Carboxylation permits the coagulation proteins to undergo a conformational change necessary both for calcium-dependent complexing of vitamin K-dependent proteins to their cofactors on phospholipid surfaces and for their biologic activity. Carboxylation of vitamin K-dependent coagulation factors is catalyzed by a carboxylase that requires the reduced form of vitamin K (vitamin KH_2), molecular oxygen, and carbon dioxide. During this reaction, vitamin KH_2 is oxidized to vitamin K epoxide, which is recycled by vitamin K epoxide reductase to vitamin K; vitamin K in turn is reduced to vitamin KH_2 by vitamin K reductase. The vitamin K antagonists exert their anticoagulant effect by inhibiting vitamin K epoxide reductase and possibly vitamin K reductase. This process leads to the depletion of vitamin KH_2 and limits the -carboxylation; as a result the function of the vitamin K-

dependent coagulant proteins (prothrombin, factor VII, factor IX, and factor X) is impaired. In addition, the vitamin K antagonists limit the carboxylation of the regulatory proteins (protein C and protein S) and thereby impair the function of these anticoagulant proteins. By inhibiting the cyclic conversion of vitamin K, oral anticoagulants cause hepatic production and secretion of partially carboxylated and decarboxylated, and therefore dysfunctional, proteins.

PHARMACOKINETICS AND PHARMACODYNAMICS

Warfarin is the most widely used oral anticoagulant in North America because its onset and duration of action are predictable and because it has excellent bioavailability. Warfarin is almost always administered orally, although an injectable preparation is available. It is rapidly absorbed from the gastrointestinal tract, reaches maximal blood concentrations in healthy volunteers in 90 minutes, and has a half-life of 36 to 42 hours. It circulates bound to plasma proteins and rapidly accumulates in the liver.

Because the dose-response relation of warfarin differs between subjects, dosage must be monitored closely. The dose response to warfarin is influenced by both pharmacokinetic factors (because of differences in absorption or metabolic clearance of warfarin) and pharmacodynamic factors (because of differences in the hemostatic response to given concentrations of warfarin). Technical factors also contribute to apparent variability

in dose response; these include inaccuracies in laboratory testing and reporting, poor individual compliance, and poor communication between individual and physician.

Drugs can influence the pharmacokinetics of warfarin by altering its metabolic clearance or reducing its absorption from the intestine. They can also influence its pharmacodynamics by inhibiting synthesis of vitamin K-dependent coagulation factors, increasing metabolic clearance of vitamin K-dependent coagulation factors (Table 3), or interfering with other pathways of hemostasis (Table 4). Many drugs influence the anticoagulant effect of warfarin through unknown mechanisms (Table 3).

Table 3

Drugs and Other Factors That Affect the Action of Warfarin

- Drugs that antagonize the anticoagulant effect of warfarin

  Impairs absorption of warfarin
  - Cholestyramine

  Increase metabolic clearance of warfarin
  - Barbiturates
  - Rifampin
  - Carbamazepine
  - Chronic alcohol use

  Mechanism unknown
  - Nafcillin
  - Sucralfate

- Drugs that potentiate the anticoagulant effect of warfarin

  Inhibit metabolic clearance of warfarin
  - Phenylbutazone
  - Sulfinpyrazone
  - Disulfiram
  - Metronidazole
  - Trimethoprim-sulfamethoxazole
  - Cimetidine
  - Oxeprazole
  - Amiodarone

  Potentiate anticoagulant effect without affecting plasma levels of warfarin (pharmacodynamic)
  - Second and third generation cephalosporins
  - Clofibrate
  - Heparin
  - Ancrod

  Mechanism not established
  - Erythromycin
  - Anabolic steroids
  - Topical testosterone
  - Ketoconazole
  - Isoniazid
  - Fluconazole
  - Piroxicam
  - Tamoxifen
  - Quinidine
  - Vitamin E (megadose
  - Phenytoin
  - Propafenone

- Factors that interfere with the effects of warfarin

  Impair effect of warfarin
  - Increased vitamin K intake (eg, leafy green salads or nutritional fluid supplements

  Potentiate effect of warfarin
  - Low vitamin K intake
  - Reduced vitamin K absorption (malabsorption states
  - Liver disease
  - Hypermetabolic states (eg, fever)

Table 4

Drugs That Potentiate the Antihemostatic Effect of Warfarin by Impairing Platelet Function

Aspirin
Other nonsteroidal anti-inflammatory drugs
Ticlopidine
Moxalactam
Carbenicillin

DRUGS THAT POTENTIATE THE ANTICOAGULANT EFFECT OF WARFARIN

Drugs that increase warfarin levels by inhibiting its metabolic clearance include phenylbutazone, sulfinpyrazone, disulfiram, metronidazole, trimethoprim-sulfamethoxazole, cimetidine, omeprazole, and amiodarone.

Drugs that increase the anticoagulant effect of warfarin without affecting its plasma levels include the second- and third-generation cephalosporins, which augment the anticoagulant effect of warfarin by inhibiting the cyclic interconversion of vitamin K; clofibrate, which by unknown mechanisms increases the anticoagulant effect of warfarin without altering its plasma concentration; heparin, which inhibits thrombin and other serine protease coagulation enzymes; and ancrod, which lowers plasma fibrinogen levels.

There is also evidence that erythromycin, anabolic steroids, and many other drugs (Table 3) potentiate the anticoagulant effect of warfarin through unknown mechanisms. Sulfonamides and broad-spectrum antibiotics can potentiate the anticoagulant effect of warfarin in individuals on a vitamin K-deficient diet by exacerbating vitamin K deficiency.

DRUGS THAT INHIBIT THE ANTICOAGULANT EFFECT OF WARFARIN

Plasma levels of warfarin are lowered and its anticoagulant effect is reduced by drugs, such as cholestyramine, that impair its absorption. Plasma levels are also lowered by drugs that increase metabolic clearance of warfarin by inducing formation of hepatic mixed oxidases, for example barbiturates, rifampicin, and carbamazepine. Chronic alcohol use may also increase the clearance of warfarin by inducing hepatic enzymes. Other drugs, such as nafcillin and sucralfate, inhibit the effect of warfarin through unknown mechanisms.

DRUGS THAT POTENTIATE THE HEMORRHAGIC EFFECT OF WARFARIN WITHOUT INFLUENCING ITS ANTICOAGULANT EFFECT

Drugs can also increase the risk of warfarin-associated bleeding by interfering with other pathways of hemostasis (Table 4). Aspirin and other nonsteroidal anti-inflammatory drugs, penicillins in high doses, and moxolactam inhibit platelet function and prolong bleeding time and therefore may increase the risk of warfarin-

associated bleeding. The effects of aspirin are particularly important because of its widespread use and prolonged effect on hemostasis and because it damages the gastric mucosa and can produce upper gastrointestinal bleeding. Serious bleeding does occur when high doses of aspirin (more than 1 g/d) and high-intensity warfarin therapy are used in combination. However, low doses of aspirin (eg, 100 mg) that have minimal gastric side effects but antithrombotic efficacy can be used with relative safety in combination with warfarin.

The pharmacodynamics of warfarin are affected by dietary and disease-related factors that can influence its anticoagulant effect. Individuals receiving long-term warfarin therapy are sensitive to fluctuating levels of dietary vitamin K, which is obtained predominantly from phylloquinone in plant material. Increased intake of dietary vitamin K sufficient to reduce the anticoagulant response to warfarin occurs in individuals on diets rich in green vegetables and those given intravenous nutritional fluid supplements high in vitamin K. The effects of warfarin can be potentiated in sick individuals with poor vitamin K intake, particularly if they are treated with antibiotics and intravenous fluids without vitamin K supplementation, and those in states of fat malabsorption. Hepatic dysfunction also potentiates the response to warfarin because of impaired synthesis of coagulation factors. Hypermetabolic states produced by fever or hyperthyroidism increase responsiveness to warfarin, probably by increasing the catabolism of vitamin K- dependent coagulation factors (Table 3).

Lists of other interacting drugs have been published. Although many of these drugs have the potential to interact with oral anticoagulants or have been reported to alter prothrombin time response to warfarin, convincing evidence that they interact with warfarin is lacking. Nevertheless, special care should be taken when treatment with any new drug is necessary in individuals who are being treated with oral anticoagulants, and prothrombin time should be monitored more frequently during the initial stages of combined drug therapy so that dose adjustments can be made when appropriate.

MONITORING ORAL ANTICOAGULANT THERAPY

The prothrombin time test is the method most commonly used for monitoring oral anticoagulant therapy. Prothrombin time increases in response to depression of three of the four vitamin K-dependent procoagulant clotting factors (prothrombin and factors VII and X). The assay is performed by adding a mixture of calcium and thromboplastin to citrated plasma (thromboplastin is a phospholipid-protein extract of tissue that contains both tissue factor and the phospholipid necessary to promote the activation of factor X by factor VII). At the beginning of anticoagulant therapy, prothrombin time primarily reflects the depression of factor VII, because this factor has the shortest half-life (5 hours) of the vitamin K-dependent factors. During maintenance therapy

the test is also sensitive to depression of prothrombin and factor X levels.

Thromboplastins vary markedly in their responsiveness, depending on tissue of origin and method of preparation, so prothrombin times determined using different reagents are not interchangeable. The "responsiveness" of a given thromboplastin is related to its potential for factor X activation; when a responsive thromboplastin is used there is a greater prolongation of prothrombin time for a given reduction in factor X. The differences in responsiveness of various thromboplastins from different manufacturers have made standardization of prothrombin time reporting difficult and exposed individuals in North America to erratic dosing.

STANDARDIZATION OF THE PROTHROMBIN TIME

The history of standardization of the prothrombin time has been reviewed. A calibration system was developed based on the linear relation between the logarithm of the prothrombin time ratios obtained with reference and test thromboplastins. This calibration model, known as the International Normalized Ratio (INR), was adopted by the World Health Organization in 1982 and is used to standardize the reporting of prothrombin time by converting the prothrombin time ratio determined with any local thromboplastin into an INR (equal to observed prothrombin time ratio, where the prothrombin time ratio is the individual's prothrombin time divided by normal pooled plasma prothrombin time and C is the International

Sensitivity Index [ISI] for each thromboplastin). The ISI is a measure of the responsiveness of a given thromboplastin to reduction of the vitamin K-dependent coagulation factors; the lower the ISI, the more responsive the reagent and the closer the derived INR will be to the observed prothrombin time ratio. The INR is the prothrombin time ratio that would be obtained if the WHO reference thromboplastin, which by definition has an ISI of 1.0, were used to assess prothrombin time. Examples of the conversion of prothrombin time ratios to corresponding INRs for thromboplastins with three different ISI values are shown in Table 5. It is clear that the same prothrombin time ratio can reflect markedly different levels of anticoagulation, depending on the ISI value. Responding to the recommendations of several international advisory groups, many manufacturers now calibrate their thromboplastin reagent against the WHO reference standard and provide the user with an ISI value, enabling hospital laboratories to report the results as INRs in a standardized manner.

Table 5

Relation Between Prothrombin Time (PT) Ratio and International Normalized Ratio (INR) for Thromboplastins With Different International Sensitivity Index (ISI) Values

Observed PT ratio	Hospital A (ISI=1.2)	Hospital B (ISI=1.8)	Hospital C (ISI=2.6)
1.3	INR=1.3/1.2 =1.4	INR=1.3/1.8 =1.6	INR=1.3/2.6 =2.0
1.5	INR=1.5/1.2 =1.6	INR=1.5/1.8 =2.1	INR=1.5/2.6 =2.9
2.0	INR=2.0/1.2 =2.3	INR=2.0/1.8 =3.5	INR=2.0/2.6 =6.1

Observed PT ratios of 1.3, 1.5, and 2.0 reflect different anticoagulant effects (represented by the INR) depending on the sensitivity (ISI) of the thromboplastin.

Although the different responsiveness of thromboplastins is a major potential cause of variability of the prothrombin time test result in individuals treated with coumarins the response is also influenced by two technical factors: the equipment used for clot detection and the magnitude of difference in ISI values between the test thromboplastin and the International Reference Preparation, which has an ISI of 1.0. When results of tests using thromboplastins with markedly different ISI values and different methods of clot detection are compared, the INR loses some precision. To overcome this problem, manufacturers are encouraged to provide ISI values adapted to the method used for clot detection.

It was reported in 1987 that the ISI values for the commercial rabbit brain thromboplastins widely used in

North America vary between 2.0 and 2.6. In a more recent study, Bussey and associates reported that the ISIs of most thromboplastins used in the United States vary between 1.8 and 2.8 and suggested that the term used in previous publications, "typical North American thromboplastin" (ISI 2.3), is no longer valid (Second ACCP Conference on Antithrombotic Therapy June 21, 1988 (1989) Chest 95(suppl):1S-169S).

It is better to use the more responsive reagents, those with a low ISI (1.0 to 1.2), particularly when low-intensity warfarin is given.

OPTIMAL THERAPEUTIC RANGE

Much progress has been made in defining the optimal therapeutic range for laboratory evaluation of oral anticoagulant therapy. The intensity of anticoagulation recommended for many indications has been reduced because of the results of randomized studies. The guidelines proposed by an advisory group of the American College of Chest Physicians in 1989 have recently undergone modification. Two levels of intensity are recommended: a less intense range corresponding to an INR of 2.0 to 3.0 and a more intense range corresponding to an INR of 2.5 to 3.5.

DOSING AND MONITORING CONSIDERATIONS

Orally administered warfarin is rapidly absorbed, but an observable anticoagulant effect is delayed until

the decarboxylated vitamin K-dependent clotting factors replace the normal clotting factors. Depending on the dose administered, the delay may range from 2 to 7 or more days. Coumarins also suppress the synthesis of carboxylated forms of protein C and protein S, natural anticoagulant proteins. Because protein C has a short half-life (similar to that of factor VII), there is the potential for an early prothrombotic effect, caused by reduced protein C activity, during the initial 24 to 48 hours of oral anticoagulant therapy. This mechanism may contribute to the coumadin-induced skin necrosis that can occur in individuals receiving warfarin who have hereditary deficiencies of protein C or protein S.

Therapy can be started with a maintenance dose or with a loading dose of approximately twice the average maintenance dose. If treatment is not urgent (eg, in individuals with chronic stable atrial fibrillation), therapy can begin with an anticipated maintenance dose of 4 to 5 mg/d; this dosage results in a steady-state anticoagulant effect in 5 to 7 days. If an antithrombotic effect is needed more urgently, heparin should be administered as indicated and warfarin administered at a loading dose of 10 mg. Heparin is discontinued when the INR is in the therapeutic range for 2 consecutive days. Because heparin may also increase the INR, a small reduction should be anticipated with some thromboplastin reagents when heparin is discontinued. Prothrombin time monitoring should be performed daily until the INR is in the therapeutic range and then three times weekly for 1 to 2 weeks, then less often, depending on the stability of

prothrombin time results. If the prothrombin time remains stable, the frequency of testing can be reduced to once every 4 weeks. If adjustments to the dose are required, the cycle of more frequent monitoring is repeated until a stable dose response is again achieved. Most individuals on long-term therapy maintain a stable dose response, but some have unexpected fluctuations in dose requirements, which can be caused by changes in diet, undisclosed drug use, poor individual compliance, surreptitious self-medication, intermittent alcohol consumption, illness, or unsuspected changes in the responsiveness of the thromboplastin used to perform the prothrombin time test. The latter problem can occur if the observed prothrombin time ratio is not converted to an INR when a individual's tests are done in different laboratories using thromboplastin reagents with different ISI values. It is important to note that different batches of thromboplastin reagents from the same manufacturer can have different ISI values.

Difficulties in monitoring oral anticoagulant therapy can usually be explained by one or more of the following factors:

- Failure to use the INR system for reporting prothrombin time results: Failure to use the INR system of reporting may result in erratic control if laboratory monitoring is done with different thromboplastin reagents that have different ISI values.

- Fluctuations in dietary vitamin K intake: Some weight reduction diets alternate periods of high vitamin K intake (eg, in green salads) and low intake. If the prescribing physician is unaware of these fluctuations, appropriate dosage adjustments will not be made and control will be erratic.

- Concomitant use of interacting drugs: Many drugs have the potential to interfere with the anticoagulant effects of coumarins. The introduction of an interacting drug or an alteration in the dosage of an interacting drug during anticoagulant therapy can result in unanticipated changes in dose-response relations.

- Poor individual compliance: Although the noncompliant individual can often be identified, in some individuals lack of compliance can be difficult to ascertain. It can be detected by assaying plasma coumarin levels.

- Inadequate laboratory monitoring. Suboptimal laboratory monitoring can occur if the period between prothrombin time testing is more than 4 weeks or if dosage change in response to a prothrombin time result outside the therapeutic range is inappropriately great.

REVERSING THE ANTICOAGULANT EFFECT OF COUMARINS

The anticoagulant effect of coumarins can be reduced or reversed by lowering the dose, stopping

treatment, administering vitamin K, or replacing the defective vitamin K-dependent coagulation factors with plasma or plasma concentrates. Reducing or stopping warfarin will usually lower the INR (without returning it to baseline) in 24 to 48 hours, depending on the INR and the dose of warfarin. This lag time reflects the time required to reduce the concentration of plasma warfarin and to replace defective vitamin K-dependent coagulation factors with newly synthesized, fully carboxylated proteins. If more rapid reversal is required, vitamin K should be administered, preferably orally or by subcutaneous injection. Intravenous infusion should be done only when a person is in shock or when bleeding is life threatening; it should be administered very slowly to minimize the risk of serious anaphylactic reaction. Very low doses of vitamin K (0.5 to 1.0 mg) should be used if moderate lowering of the INR is required (for example, from 2.5 to 1.5 before elective surgery), particularly if warfarin will be continued in the immediate future. Higher doses of vitamin K (5.0 to 10.0 mg) should be used if a more rapid effect or complete reversal of the anticoagulant effect is required. Doses of vitamin K of 5 mg or more may render the individual resistant to warfarin for up to 1 week. With parenteral vitamin K administration, a partial reversal of the anticoagulant effect of warfarin is evident within 6 hours. In the case of warfarin overdose, vitamin K administration may have to be repeated because the vitamin is cleared from the circulation more rapidly than is warfarin. If reversal of the anticoagulant effect is urgent because of serious

overdose or life-threatening bleeding, high-dose vitamin K (10 mg repeated as necessary) should be supplemented by plasma or plasma concentrates containing the vitamin K-dependent clotting factors.

CLINICAL RESULTS

The clinical effectiveness of coumarins for a variety of indications has been established in well-designed studies. Two levels of anticoagulant intensity have been compared in clinical trials in individuals with venous thrombosis or with tissue or mechanical heart valves. These studies have shown that a less intense regimen (INR, 2.0 to 3.0) is not only as effective as a high-intensity regimen (INR, 3.0 to 4.5) but much safer.

Oral anticoagulants are effective in the primary and secondary prevention of venous thromboembolism. They are also effective in the prevention of systemic arterial embolism in individuals with atrial fibrillation or with tissue or mechanical prosthetic heart valves; of acute myocardial infarction in individuals with peripheral arterial disease; and of stroke, recurrent infarction, and death in individuals with acute myocardial infarction. Treatment with oral anticoagulants is also indicated in individuals with valvular heart disease or dilated cardiomyopathy to prevent systemic arterial embolism, although its effectiveness has never been demonstrated in a randomized clinical trial. For most indications, a moderate anticoagulant effect with a target INR of 2.0 to 3.0 (less intense regimen) is appropriate.

PREVENTION OF VENOUS THROMBOEMBOLISM

Oral anticoagulants are effective in preventing venous thrombosis after hip surgery and major general surgery at a target INR of 2.0 to 3.0, both when started before surgery or on the first postoperative day. The risk of clinically important bleeding with this relatively low-intensity regimen is small, but because warfarin prophylaxis is more complicated than fixed low-dose heparin, it is generally reserved for very high-risk individuals such as those with previous venous thrombosis or those undergoing major orthopedic procedures.

TREATMENT OF DEEP VEIN THROMBOSIS

It is standard practice to treat individuals who have venous thrombosis or pulmonary embolism with heparin for 5 to 10 days and then with a coumarin derivative (usually warfarin) for 3 to 6 months. There is evidence from randomized trials that oral anticoagulants are effective in preventing recurrent venous thromboembolism when administered after a course of heparin therapy and that a less intense regimen (INR, 2.0 to 3.0) is as effective but much safer than a more intense regimen (INR, 3.0 to 4.5).

The effectiveness of coumarins in the secondary prevention of venous thrombosis and pulmonary embolism was suggested by the results of a prospective cohort study (Goldberg *et al.* (1985) Am Heart J 109(part 1):616-622)

and then demonstrated by the results of two more randomized studies (Hull *et al.* (1979) N Engl J Med 301:855-858; Lagerstedt *et al.* (1985) Lancet 2:515-518). In the first randomized trial (Hull *et al.* (1979) N Engl J Med 301:855-858), individuals with venographically confirmed proximal deep vein thrombosis were treated with an initial course of heparin and then randomly assigned to groups receiving either sodium warfarin (INR, 2.5 to 4.5) or fixed low-dose subcutaneous heparin (5,000 units every 12 hours); both treatments were for 3 months. Low-dose subcutaneous heparin was ineffective; the rate of recurrent venous thromboembolism was 47% in the 19 individuals in this group and 0% in the 17 individuals treated with sodium warfarin. This study therefore provided evidence that individuals with deep vein thrombosis who are treated with an initial course of heparin therapy have a high risk of recurrence unless effective anticoagulant therapy is continued for a period of months after discharge from the hospital. These conclusions are supported by a second study in individuals with symptomatic, venographically confirmed calf vein thrombosis who were randomly assigned to groups receiving either heparin for 5 days followed by warfarin for 3 months (23 individuals) or only heparin for 5 days (28 individuals). There were no recurrences in individuals who were treated with warfarin but a recurrence rate of 29% in the control group (Lagerstedt *et al.* (1985) Lancet 2:515-518).

The intense oral anticoagulant regimen (INR, 2.5 to 4.5) of one study was associated with a 21% rate of

clinically overt bleeding (Hull *et al.* (1979) N Engl J Med 301:855-858). In contrast, in another study (Leizorovicz and Boissel (1983) Eur J Clin Pharmacol 24:333-336) the subcutaneous heparin adjusted to maintain the midinterval activated partial thromboplastin time (determined 6 hours after injection) at 1.5 times the control value produced less bleeding but was equally effective. Therefore, a randomized trial was performed to determine whether a less intense warfarin regimen would be effective in preventing recurrent venous thrombosis but have a lower risk of bleeding than high-intensity therapy. The high-intensity therapeutic range (INR, 2.5 to 4.5) was compared with one less intense (INR, 2.0 to 2.5), and it was demonstrated that the less intense regimen was as effective as the more intense regimen (the incidence of recurrence was approximately 2% in both groups), but the incidence of bleeding was 4.2% and 22.4%, respectively.

The optimal duration of oral anticoagulant therapy has been addressed by three studies, the results of which were inconclusive. The currently recommended approach is to treat all individuals having a first episode of proximal venous thrombosis with an initial course of heparin and then to continue oral anticoagulants for 3 months if there is no continuing risk factor or indefinitely if there is one.

MYOCARDIAL INFARCTION

The role of oral anticoagulants in the treatment of acute myocardial infarction remains controversial. The

thromboembolic complications of acute myocardial infarction are caused by (1) the acute ischemic effects of thrombotic coronary occlusion that result in myocardial necrosis, (2) systemic embolism secondary to intracardiac thrombosis in individuals with anterior wall myocardial infarction or with myocardial infarction associated with atrial fibrillation or congestive cardiac failure, and (3) venous thromboembolism in bedridden individuals.

There is evidence that less intense warfarin therapy (INR, 2.0 to 3.0) is effective in preventing stroke and venous thromboembolism in individuals with acute myocardial infarction; high-intensity warfarin therapy (INR, approximately 3.0 to 4.5) has been shown to prevent recurrent infarction, stroke, and death.

Evidence that oral anticoagulants are effective in the early treatment of acute myocardial infarction first came in the 1960s and 1970s from studies indicating that a low-intensity warfarin regimen (presumptive INR, 1.5 to 2.5) is effective in preventing stroke and pulmonary embolism. The effectiveness of oral anticoagulants in individuals with acute myocardial infarction was evaluated in three randomized trials; in two of these, the Medical Research Council study and the Veterans Administration Cooperative Study (Br Med J. (1969) 1:335-342; JAMA (1973) 225:724-729), a significant reduction in stroke was demonstrated, but in the third, the Bronx Municipal Study (Drapkin and Merskey (1972) JAMA 222:541-548), a nonsignificant trend was reported. In the Bronx Municipal Study there was a significant reduction in mortality in

the group given heparin followed by phenindione (compared with the group given minimal phenindione), but in the other two studies there was no effect of oral anticoagulation therapy on mortality. There was a reduction in the incidence of clinically diagnosed pulmonary embolism in all three studies. The findings are supported by the overviews by Chalmers and associates (Br Med J (1969) 1:335-342) and McMahon and associates (Cairns *et al.* (1992) Chest 102(suppl):456S-481S) and by autopsy studies demonstrating that anticoagulant treatment reduces both mural thrombosis and systemic embolism.

Evidence that oral anticoagulants are effective in the long-term management of acute myocardial infarction comes from analysis of pooled data from seven randomized trials published between 1964 and 1980. Oral anticoagulant therapy during a 1- to 6-year treatment period reduced the combined incidence of mortality and nonfatal reinfarction by approximately 20%.

The value of oral anticoagulants in the long-term treatment of myocardial infarction is supported by the results of two studies. The Sixty-Plus Reinfarction Study Group (Lancet (1980) 2:989-994) limited their subject population to individuals over the age of 60 who had been treated with oral anticoagulants for at least 6 months. Although there were significant reductions in reinfarction and stroke in individuals randomly assigned to receive continuing anticoagulant therapy, the findings were limited by the study's lack of generalizability as a "stopping trial" in a select age group. There was no age

restriction in the WARIS study by Smith and associates (Smith *et al.* (1990) N Engl J Med 323:147-152), the results of which attracted considerable attention because of the 50% reduction in the incidence of the combined outcomes of recurrent infarction, stroke, and mortality. In both studies higher-INR regimens (2.7 to 4.5 and 2.8 to 4.8, respectively) were used, and in both there was an increased incidence of bleeding with anticoagulants. Indirect support for the efficacy of oral anticoagulants in individuals with coronary artery disease comes from a randomized trial of individuals with peripheral arterial disease; compared with an untreated control group, a relatively high-intensity oral anticoagulant regimen (INR, 2.6 to 4.5) produced a significant reduction in mortality from 6.8% to 3.3% per year.

Since 1982 there have been a number of reports on the risk of systemic embolism in individuals with acute myocardial infarction. Four studies (Steffensen (1969) Acta Med Scand 186:519-521; Hilden *et al.* (1961) Lancet 2:327-331; Weinreich *et al.* (1984) Ann Intern Med 100:789-794; Ezekowitz *et al.* (1984) Chest 86:35-39) identified the embolic risk in anterior acute myocardial infarction and three reported on the frequency of associated mural thrombosis detected by echocardiography (Steffensen (1969) Acta Med Scand 186:519-521; Hilden *et al.* (1961) Lancet 2:327-331). Only two (Steffensen (1969) Acta Med Scand 186:519-521; Ezekowitz *et al.* (1992) N Engl J Med 327:1406-1412) included sufficient numbers of individuals to provide narrow confidence intervals on the observed rates; according to these two studies, the risk of stroke

is likely to be between 2% and 6% for individuals with anterior acute myocardial infarction.

It has been recommended on the basis of these observations that warfarin treatment (INR, 2.0 to 3.0) for up to 3 months be considered to prevent stroke in individuals with Q-wave acute anterior myocardial infarction. The role of warfarin in long-term treatment to prevent reinfarction and the possibility of combining low-intensity warfarin with low-dose aspirin are currently being explored.

PROSTHETIC HEART VALVES

Individuals with prosthetic heart valves are at risk of systemic embolism, the most common clinical manifestation of which is stroke. The risk of systemic embolism is greater with mechanical than with bioprosthetic valves and with prosthetic mitral than with aortic valves; risk also increases if there is associated atrial fibrillation. For individuals with tissue prosthetic valves who are in sinus rhythm, the risk of embolism is largely limited to the first 3 months after valve insertion; for individuals with mechanical prosthetic valves (particularly in the mitral position), the risk of systemic embolism is lifelong.

MECHANICAL PROSTHETIC VALVES

In randomized trials warfarin has been effective in reducing the risk of systemic embolism in individuals

with mechanical prosthetic valves when given at a lower intensity than has been used in the past. For this reason, a target INR of 2.5 to 3.5 is recommended for these individuals. If embolic complications occur at the recommended intensity, then either 160 mg/d aspirin or 400 mg/d dipyridamole should be prescribed in addition to warfarin because there is recent evidence that aspirin augments the effects of warfarin when so used. Low-dose aspirin is less expensive and has fewer side effects than dipyridamole. If individuals are at high risk of bleeding, the target therapeutic range can be reduced to an INR of 2.0 to 3.0, with or without the addition of dipyridamole.

BIOPROSTHETIC VALVES

The risk of thromboembolism is less with uncomplicated bioprosthetic valves than with mechanical valves. Warfarin is as effective, but much safer, when used at a target INR of 2.0 to 3.0 than at an INR of 3.0 to 4.5. Consequently, the less intense therapeutic range of 2.0 to 3.0 is indicated. The risk of thromboembolism is limited mainly to the first 3 months after surgery in the individual with no complications but is present indefinitely in the individual with atrial fibrillation. Therefore, in individuals with mitral bioprosthetic valves who have no complications, coumarin treatment is limited to 3 months. Longer-term therapy is indicated for individuals with atrial fibrillation, those with an atrial thrombus detected on echocardiography, and those who

develop a systemic embolus. Individuals with uncomplicated bioprosthetic valves in the aortic position are at very low risk of systemic embolism and therefore do not require anticoagulant therapy except for the first 3 months after surgery. Extrapolating from the results of studies of nonvalvular atrial fibrillation, it would be reasonable to treat these individuals with aspirin, even though there is no direct evidence that aspirin is an effective preventive measure against thromboembolism.

ATRIAL FIBRILLATION

Individuals with nonvalvular atrial fibrillation are at increased risk of stroke, with an overall incidence of 5% per year. Risk of stroke increases with increasing age and with the associated cardiac disorders of previous myocardial infarction, angina, history of heart failure, left atrial dilatation, left ventricular dysfunction, mitral calcification, history of hypertension, and history of previous thromboembolism. Conversely, the risk of stroke is very low (<2% per year) in individuals less than 60 years old who have no associated cardiac abnormality (lone atrial fibrillation). Individuals with intermittent atrial fibrillation have the associated risk of stroke.

In five randomized studies that included untreated control groups (there was also an aspirin-treated group in two), it was reported that low-intensity oral anticoagulant therapy is effective in reducing risk of thromboembolic stroke in individuals with nonvalvular atrial fibrillation (Ezekowitz *et al.* (1992) N Engl J Med

327:1406-1412; Turpie *et al.* (1988) Lancet 1:1242-1245; Saour *et al.* (1990) N Engl J Med 322:428-432; Altman *et al.* (1991) J Thorac Cardiovasc Surg 101:427-431; Kretschmer *et al.* (1988) Lancet 1:797-799). Based on the results of these five studies, it has been recommended that warfarin should be used in individuals with nonvalvular atrial fibrillation if they have an associated cardiac abnormality or if they are over the age of 60 years and have no contraindications to warfarin. Aspirin is reported to be effective in reducing risk of thromboembolic stroke, though less so than warfarin. Therefore, aspirin should be considered for individuals at high risk for stroke when warfarin is contraindicated, although these recommendations may have to be modified on the basis of subgroup analysis of the SPAF I study (Turpie *et al.* (1988) Lancet 1:1242-1245) and the recently completed SPAF II study (Lancet (1980) 2:989-994; Ann Intern Med (1992) 116:1-5). The recent evidence indicates that individuals under the age of 75 who are free of clinical risk factors (associated heart disease, history of hypertension, or previous stroke) and echocardiographic risk factors (left ventricular global dysfunction or left atrial enlargement) are at low risk for stroke development (approximately 1% per year). The use of aspirin rather than warfarin is not unjustified in this group.

The risk of stroke in individuals with atrial fibrillation is increased when they have associated valvular heart disease; because there is strong evidence that anticoagulants are effective in reducing the risk of

stroke, these individuals should be given anticoagulant therapy.

The risk of systemic embolism is increased during electrical cardioversion, but is reduced if prophylactic warfarin is used.

VALVULAR HEART DISEASE

MITRAL VALVE PROLAPSE

Mitral valve prolapse is a common disorder, occurring in 4% to 6% of asymptomatic individuals. In most cases it is benign, and uncomplicated cases require no antithrombotic treatment. However, treatment with antithrombotic agents is indicated if embolic complications occur or if there is associated atrial fibrillation.

PARADOXICAL EMBOLISM

In recent years there have been a number of reports of an association between patent foramen ovale and stroke in young subjects. Although the risk of stroke in subjects with patent foramen ovale is unknown, identification of the defect in young individuals with stroke from no other apparent cause is an indication for either closure of the defect or long-term anticoagulant therapy.

RHEUMATIC MITRAL VALVE DISEASE

Individuals with rheumatic mitral valve disease are at increased risk of systemic embolism, and results of descriptive studies suggest that warfarin reduces this risk. The risk of systemic embolism is moderate even in an individual who is in sinus rhythm and whose left atrium is of normal size. In such individuals, the risk of serious bleeding must be balanced by the risk of embolism. The risk of embolism increases if there is associated atrial fibrillation, previous systemic embolism, or enlarged left atrial diameter; warfarin (INR, 2.0 to 3.0) is indicated in case of any of these complications. If recurrent embolism occurs despite adequate warfarin treatment (eg, INR >2.0), the therapeutic INR range can be increased to 2.5 to 3.5 or, alternatively, 160 to 325 mg aspirin per day can be added to the warfarin.

DILATED CARDIOMYOPATHY

There is a high prevalence of right and left ventricular mural thrombi in individuals with dilated cardiomyopathy. Factors predisposing to thrombosis include stasis and low shear rates in the hypokinetic ventricle. In a retrospective survey the rate of systemic embolism in individuals not treated with anticoagulants was 3.5%, and there were no cases of systemic embolism in a historic comparative group. Based on this limited evidence, as well as on the proven effectiveness of low- to moderate-intensity anticoagulant therapy in nonvalvular atrial fibrillation and the absence of any truly

randomized studies, it would be reasonable to recommend long-term, low-intensity (INR, 2.0 to 3.0) anticoagulant therapy for individuals with dilated cardiomyopathy.

ADVERSE EFFECTS

Bleeding is the main complication of oral anticoagulant therapy. The risk of bleeding is influenced by the intensity of anticoagulant therapy and is reduced dramatically by lowering the INR range from 3.0-4.5 to 2.0-3.0 (Table 6). Although this difference in anticoagulant intensity is produced by a reduction of the dose of warfarin by only approximately 1 mg, the effect on bleeding is profound. The rate of oral anticoagulant-induced bleeding is increased by the concomitant use of high doses of aspirin, which both impair platelet function and produce gastric erosions. The risk of bleeding is increased in those who are more than 65 years old, have a history of stroke or gastrointestinal bleeding, or serious comorbid conditions such as renal insufficiency or anemia (Cairns *et al.* (1992) Chest 102(suppl):456S-481S; Fuster *et al.* (1981) Am J Cardiol 47:525-531). Bleeding that occurs when the INR is less than 3.0 is frequently associated with an obvious underlying cause or an occult gastrointestinal or renal lesion.

Table 6
Relation Between Bleeding and Intensity of Anticoagulant Therapy

Study	Individuals (n)	Duration of anticoagulant therapy	Therapeutic range of INR	Percentage of individuals who bled	P
Hull *et al* (1979) N Engl J Med 301:855-858	96	3 mo	2.5-4.1	22.4	.015
(Deep vein thrombosis)			2.0-2.3	4.3	
Turpie *et al*(1988) Lancet 1:1242-1245	210	3 mo	2.5-4.9	13.9	<.002
(Prosthetic heart valves, tissue)			2.9-2.3	5.9	
Saour *et al* (1990) N Engl J Med 322:428-432	247	3.5 y	7.4-10.8	42.4	<.002
(Prosthetic heart valves, mechanical)			1.9-3.6	21.3	
*Altman *et al* (1991) J Thorac Cardiovasc Surg 101:427-431	99	11.2 mo	3.0-4.5	24.0	<.02
(Prosthetic heart valves, mechanical)			2.0-2.9	6.0	

INR indicates International Normalized Ratio.
*Individuals also given 300 mg aspirin and 75 mg dipyridamole twice a day.

SKIN NECROSIS

The most important nonhemorrhagic side effect of warfarin is skin necrosis. This uncommon complication is usually observed on the third to eighth day of therapy and is caused by extensive thrombosis of the venules and capillaries within the subcutaneous fat. Associations have been reported between warfarin-induced skin necrosis and deficiency of protein C or, less commonly, of protein S, but this complication can also occur in people who do

not have the deficiency. A role for protein C deficiency seems probable and is supported by the similarity of the lesions to those seen in neonatal purpura fulminans, which complicates homozygous protein C deficiency. The reason for the unusual localization of the lesions to subcutaneous fat deposits is unknown. The optimal anticoagulant treatment regimen for individuals with protein C or protein S deficiency is also unknown; a reasonable empirical approach is to start with an initial course of heparin, begin warfarin at a maintenance dose of 5 mg, and give both anticoagulants in combination for approximately 7 days.

The management of individuals with warfarin-induced skin necrosis who require anticoagulant therapy for an indefinite period is problematic. These individuals can be treated with subcutaneous heparin for the long term, but this is inconvenient and carries a risk of osteoporosis. It might be safe to reintroduce warfarin in very low doses initially, in combination with heparin, and use combined treatment for 10 to 14 days, during which time the warfarin dose is gradually increased. It should be noted, however, that heparin treatment may not stop coumarin necrosis.

In addition, the present invention may further include the use of indirect phenotyping to identify individuals with a particular genotype, which is associated with extremely high risks of toxicity from a particular anticoagulant. According to one embodiment of the present invention, those individuals without the "high

risk" genotype will be phenotyped and dosed according to their individual molar ratio, while the high risk individuals will not be recommended for treatment with that particular anticoagulant. By employing genotyping in combination with phenotyping to screen individuals for treatment with anticoagulants, those individuals found to be carrier of a high risk genotype can be eliminated as candidates for such treatment without the necessity of phenotyping.

The integration of phenotyping tests into the drug development process provides for a decreased number of individuals participating in a drug treatment testing trial, as individual screening using phenotyping can be conducted prior to the trial to select those individuals displaying the capability to metabolize the drug of interest safely and effectively. In particular, those individuals identified as being metabolically incompatible with the drug treatment trial can be screened out before undergoing treatment with the drug. This aspect of the present invention provides a means to selectively treat only those individuals identified as having the ability to safely metabolize the drug. In addition, the decrease in individual number will result in decreased costs and allow the drug to reach the market faster. In addition, the clinical use of a phenotypic screening method of the present invention provides the ability to individualize treatments according to phenotypic profiles. In particular, dose specific determinations corresponding to a calculated rate of metabolism for that drug phenotype is possible on an individual basis.

Pre-trial screening would involve the phenotyping of all individuals prior to inclusion in the trial. The phenotype status could then be used to identify those individuals at high risk for serious adverse events (SAEs) and ensure that they were not included in the trial. The remaining individuals would then be treated with drug doses customized in correlation to their level of CYP2C9 activity, in the case of anticoagulant drugs. The customized dose would ensure that the individuals were receiving a safe efficacious treatment, corresponding to their ability to safely metabolize the drug. Similarly, according to the present invention, individualized treatment has application in the clinical environment where drug treatment dosages will be customized according to an individual's phenotypic profile or calculated rate of metabolism.

According to the present invention, phenotypic determinants for one or more of the following enzymes may be characterized to provide a phenotypic profile on an individual basis:

CYP3A4

The CYP3A family constitutes approximately 25% of the total CYP 450 enzymes in the human liver.

POLYMORPHISM

A large degree of inter-individual variability in the expression of the CYP3A4 isoenzymes has been shown in

the human liver (>20 fold). However, the activity of CYP3A4 metabolism is distributed unimodally and as a result, there are currently no categorical classifications for distinct subsets of this population. Further, there is currently no evidence of a common allelic variant in the coding region of the gene. Recently, a rare allelic variant was identified in exon 7 (CYP3A4*2). Limited data suggested that this mutation may result in altered substrate dependent kinetics compared with the wt CYP3A gene. It has been considered that the large inter-individual variability in the activity of CYP3A may reflect differences in transcriptional regulation. Another allelic variant in the 5'-flanking region of CYP3A has been identified (CYP3A4*1B) that involves an A→G transition at position -290 from the transcriptional initiation site. It has been speculated that this nucleotide substitution may be associated with a reduced level of CYP3A activity. Ongoing studies are investigating the existence of a common allelic variant linked to CYP3A4 activity.

CYP3A4 metabolizes several drugs and dietary constituents including delavirdine, indinavir, ritonavir, saquinavir, amprenavir, zidovidine (AZT), nelfinavir mesylate, efavirenz, nevirapine, imiquimod, resiquimod, donezepil, lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin, rosuvastatin, benzafibrate, clofibrate, fenofibrate, gemfibrozil, niacin, benzodiazepines, erythromycin, dextromethorphan

dihydropyridines, cyclosporine, lidocaine, midazolam, nifedipine, and terfenadine.

In addition, CYP3A4 activates environmental pro-carcinogens especially N'-nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3- pyridyl- 1 -butanone) (NNK), 5-Methylchrysene, and 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products).

INDUCTION AND INHIBITION

CYP3A4 is induced by a number of drugs including dexamethasone, phenobarbital, primidone and the antibiotic rifampicin. Conversely, CYP3A4 is inhibited by erythromycin, grapefruit juice, indinavir, ketoconazole, miconazole, quinine, and saquinavir.

INTER ETHNIC DIFFERENCES

Several studies have suggested that the activity of CYP3A4 varies between populations. Plasma levels of a CYP3A4 substrate drug after oral administration were reported to be twofold to threefold higher in Japanese, Mexican, Southeast Asian and Nigerian Populations compared with white persons residing in various countries. In addition, the CYP3A4*1B allele has been reported to be more frequent in African-American populations as compared to European Americans or Chinese populations (66.7% vs. 4.2% vs. 0%, respectively). The rare CYP3A4*2 allele was found in 2.7% of a white population and was absent in the black and Chinese individuals. It is reasonable that, in

drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

Due to the variability in CYP3A4 activity within the population it would be advantageous to be provided with a system and method for quickly and easily determining an individual's CYP3A4 metabolic phenotype prior to administering a CYP3A4-dependant treatment thereto. In particular, such a system and method are believed to have enormous benefit in the individualization of therapy, and in particular with respect to the individualization of therapy with many hyperlipidia agents, including HMG-CoA reductase inhibitors (statins), fibrates, bile acid sequestrants and nicotinic acid (niacin).

CYCLOSPORINE

An example of the need for phenotyping in drug dosing is the case of cyclosporine in the treatment of organ transplant individuals. Cyclosporine is an immunosuppressant agent (drug) administered post transplant to protect the new organ from being rejected. Plasma levels of this drug are critical as high levels lead to renal toxicity but low levels can lead to organ rejection. Cyclosporine is metabolized via the CYP3A4 system. Several studies have indicated the importance of monitoring CYP3A4 activity in maintaining an effective and

safe cyclosporine dose. For these reasons, the utility of a reliable phenotyping test for CYP3A4 is evident.

DIRECT PHENOTYPIC DETERMINANTS OF CYP3A4

Different probe substrates can be used to determine the CYP3A4 phenotype (dapsone, testosterone, nifedipine, midazolam, erythromycin, dextromethorphan, cortisol). In accordance with the present invention, suitable probe substrates include without limitation, midazolam, dextromethorphan, erythromycin, dapsone, testosterone, nifedipine and cortisol.

Of these midazolam is the preferred probe substrate. The structures of midazolam and its hydroxylated metabolite, 1'-hydroxymidazolam are illustrated in Fig. 1. In accordance with the present invention, the molar ratio of midazolam and its metabolite is used to determine the CYP3A4 phenotype of the individual as follows:

$$\frac{\text{1'-hydroxymidazolam}}{\text{midazolam}}$$

An individual's ratio will be considered as indicative of CYP3A4 enzyme activity with a lower ratio indicating poorer metabolism and a higher ratio indicating more extensive metabolism. The activity of CYP3A4 metabolism is distributed unimodally and hence no antimode is present. The levels of CYP3A4 activity as determined by direct phenotyping will be used.

INDIRECT PHENOTYPIC DETERMINANTS OF CYP3A4 (GENOTYPING)

To date only two mutant alleles have been identified for the CYP3A4 gene (CYP3A4*1B and CYP3A4*2). Studies have been unable to correlate these mutations with the large inter-individual variation in CYP3A4 activity. Despite confirmation in this regard to date, the use of indirect phenotyping is contemplated in accordance with the present invention. Ongoing studies continue to investigate this aspect of the present invention.

NAT2

POLYMORPHISM

Individuals are genetically polymorphic in their rate of N-acetylation of drugs via the N-acetyltransferase (NAT2) pathway (Meyer, U.A. (1994) Proc. Natl. Acad. Sci. USA, 91:1983-1984). Two major metabolic phenotypes can be distinguished: fast and slow N-acetylators. Drugs that are individual to N-acetylation polymorphism include sulfonamides (sulfamethazine), antidepressants (phenelzine), antiarrhymics (procainamide), and antihypertensives (hydrazine). Some adverse therapeutic consequences of the acetylator phenotype are peripheral neuropathy and hepatitis. In an opposite manner, the N-acetylation of procainamide produces a therapeutically active metabolite with reduced toxicity. N-acetylation polymorphism has also been linked to the detoxification

pathway of some environmental carcinogenic arylamines and there is a higher frequency of bladder cancers among chemical dye workers who are slow N-acetylators.

The NAT2 gene is polymorphic, there have been 9 mutations detected and 14 mutant alleles. Six mutant alleles are responsible for 99% of Caucasian slow acetylators (NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6A, NAT2*7B, and NAT2*13). The NAT2*4 allele is the wild-type allele.

INTER ETHNIC DIFFERENCES

The frequencies of PM (poor metabolizer) and EM (extensive metabolizers) (autosomal recessive trait) show considerable inter ethnic differences for the N-acetylation polymorphism. In Caucasians, the frequencies are approximately 60 and 40%, respectively, while in Orientals, they are 20 and 80%, respectively (Meyer, U.A. (1994) Proc. Natl. Acad. Sci. USA, 91:1983-1984). It is reasonable that, in drug metabolism studies, each ethnic group is studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

DIRECT PHENOTYPING - PHENOTYPIC DETERMINANTS OF NAT2

Different probe substrates can be used to determine the NAT2 phenotype. In accordance with the present invention a suitable probe substrate is, without limitation caffeine. Caffeine is widely consumed and relatively safe. A phenotype may be generally determined

from ratios of the caffeine metabolites 5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU) and 1-methylxanthine (1X) present in urine samples of an individual collected after drinking coffee. The structures of these metabolites are illustrated in Fig. 2. The ratio of these metabolites provides a determination of an individual's N-acetylation (NAT2) phenotype.

AAMU (or AFMU) / 1X

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the acetylation phenotype of the individual as follows. Individuals with a ratio less than 1.80 are slow acetylators.

INDIRECT PHENOTYPING (GENOTYPING)

An example of NAT2 genotyping involves the amplification of a 547 bp fragment which includes the 5 of the 6 mutant alleles which are responsible for 99% of Caucasian slow acetylators. Analysis of these 5 alleles and the wt allele can be performed by examining 4 mutations (Smith CAD *et al.* J Med Genet (1997) 34:758-760).

The PCR amplification is performed with the following primers:

5'-GCTGGGTCTGGAAGCTCCTC-3' (SEQ ID NO:1)

5'-TTGGGTGATACATACACAAGGG-3' (SEQ ID NO:2)

The analysis of this fragment with 4 restriction digestion enzymes allows the detection of 6 alleles (NAT2*4 (wt) and the mutants NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6 and NAT2*7). Each of the 6 alleles have distinct combinations of the mutations and as each mutation alters a specific restriction digestion enzyme site (*Kpn*I, *Dde*I, *Taq*I or *Bam*HI), the performance of 4 separate digestions of the 547 bp fragment will allow the identification of the different alleles.

CYP1A2

CYP1A2 constitutes 15% of the total CYP 450 enzymes in the human liver.

POLYMORPHISM

CYP1A2 may be polymorphic although it remains to be established firmly. To date no mutant alleles have been identified. Three metabolic phenotypes can be distinguished: rapid, intermediate and slow metabolizers. CYP1A2 metabolizes several drugs and dietary constituents including resiquimod, imiquimod, tacrine, acetaminophen, anti pyrine, 17 β-estradiol, caffeine, cloipramine, clozapine, flutamide (antiandrogenic), imipramine, paracetamol, phenacetin, tacrine and theophylline.

In addition, CYP1A2 activates environmental pro-carcinogens, especially heterocyclic amines and aromatic amines. In one study it has been shown that individuals who are fast N-acetylators and have high CYP1A2 activity are at a greater risk for colorectal cancer (35% of cases vs. 16% of controls, OR=2.79 (P=0.00-2).

INDUCTION AND INHIBITION

CYP1A2 is induced by a number of drugs and environmental factors such as omeprazole, lansoprasole, polyaromatic hydrocarbons and cigarette smoke. CYP1A2 is inhibited by oral contraceptives, ketoconazole, α-napthoflavone, fluvoxamine (serotonine uptake inhibitor), and furafylline.

INTER ETHNIC DIFFERENCES

The activity of CYP1A2 varies broadly (60 to 70 fold) in a given population. Slow, intermediate and rapid CYP1A2 phenotypes have been distinguished. The proportion of these three CYP1A2 phenotypes varied between ethnic groups and countries: % of intermediates: 50, 70, 60, >95, 60, 20 in U.S.A., African-American, China, Japan, Italy and Australia, respectively. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

THEOPHYLLINE

A classical example of the need for phenotyping in drug dosing is the case of theophylline. Theophylline is used in the treatment of asthma. However, theophylline toxicity continues to be a common clinical problem, and involves life-threatening cardiovascular and neurological toxicity. Theophylline is cleared from the body via the CYP1A2 metabolizing system. Inhibition of CYP1A2 by quinolone antibiotic agents or serotonine reuptake inhibitors may result in theophylline toxicity. For these reasons, the utility of a reliable phenotyping test for CYP1A2 is evident.

DIRECT PHENOTYPIC DETERMINANTS OF CYP1A2

Different probe substrates can be used to determine the CYP1A2 phenotype (caffeine, theophylline). In accordance with the present invention suitable probe substrates include without limitation, caffeine, theophylline or acetaminophen.

Of these caffeine is the preferred probe substrate. Caffeine is widely consumed and relatively safe. The structure of caffeine and its metabolites 1,7-dimethylxanthine (1,7 DMX) and 1,7-dimethyluric acid (1,7 DMU)are illustrated in Fig. 3.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the CYP1A2 phenotype of the individual as follows:

1,7-dimethylxanthine (1,7 DMX) +
1,7-dimethyluric acid (1,7 DMU) /caffeine

Molar ratios of 4 and 12 separate slow, intermediate and fast CYP1A2 metabolizers, respectively (Butler *et al.* (1992) Pharmacogenetics 2:116-117).

INDIRECT PHENOTYPIC DETERMINANTS OF CYP1A2 (GENOTYPING)

To date no mutant alleles have been identified for the CYP1A2 gene. Therefore, indirect phenotyping is not currently possible for CYP1A2.

NAT1

The NAT1 enzyme catalyzes the N-acetylation of many compounds. It is expressed in the liver as well as in mononuclear leucocytes.

POLYMORPHISM

The NAT1 gene was for a long time classified as monomorphic. However, it is now suggested that NAT1, like the other N-acetyltransferase gene (NAT2), is polymorphic. Studies have demonstrated the presence of one wild type allele (NAT1*4) and six mutant alleles (NAT1*3, NAT1*5, NAT1*10, NAT1*11, NAT1*14 and NAT1*17). NAT1 has two phenotypes: slow and rapid acetylators (e.g. NAT1*4 vs. NAT1*10 genotypes respectively).

NAT1 metabolizes several drugs and dietary constituents including p-aminobenzoic acid, p-aminosalicylic acid, and dapsone.

In addition, NAT1 activates environmental pro-carcinogens, especially diaminobenzidine, N-hydroxy-4-aminobiphenyl, and heterocyclic aromatic amines (MeIQx and PhIP). In one study it has been shown that individuals who have the NAT1*10 allele, and hence are rapid N-acetylators, are at a greater risk for colorectal cancer (OR=1,9; 95% CI=1.2-3.2), while in another study they have an increased risk for bladder cancer (metabolize benzidine).

INTER ETHNIC DIFFERENCES

The activity of NAT1 varies broadly in a given population. Slow, and rapid NAT1 phenotypes have been distinguished. The NAT1*10 genotype that is associated with rapid metabolic phenotype was monitored in three different ethnic populations, Indian, Malaysian and Chinese. The frequency of NAT1*10 allele was 17%, 39% and 30%, respectively. The NAT1*4 genotype, associated with slow metabolizers, had a frequency in the same populations of 50%, 30% and 35%, respectively. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

DAPSONE

A classical example of the need for phenotyping in drug dosing is the case of dapsone. Dapsone is used in the treatment of malaria and is being investigated for the treatment of *Pneumocystis carinii* pneumonia in AIDS individuals. Adverse effects include rash, anemia, methemoglobinemia, agranulocytosis, and hepatic dysfunction. Dapsone is cleared from the body via the NAT1 metabolizing system. A study has shown a correlation between slow acetylation and increased adverse reactions to dapsone (46% vs. 17% for slow and fast acetylators, respectively). For these reasons, the utility of a reliable phenotyping test is evident.

PHENOTYPIC DETERMINANTS OF NAT1

Different probe substrates can be used to determine the NAT1 phenotype, such as (p-aminosalicylic acid (pASA), and p-aminobenzoic acid (pABA)). In accordance with the present invention suitable probe substrates include, with out limitation, p-aminosalicylic acid, and p-aminobenzoic acid.

Of these pASA is the preferred probe substrate. The structure of pASA and its acetylated metabolite p-acetylaminosalicylic acid are illustrated in Fig. 4.

In accordance with the present invention, the molar ratio of pASA and its acetylated metabolite is used to determine the NAT1 phenotype of the individual as follows:

pASA

pAcetyl-ASA

INDIRECT PHENOTYPIC DETERMINANTS OF NAT1 (GENOTYPING)

The NAT1 alleles NAT1*4 (wt) and the mutant NAT1*14 can be determined either by PCR-RFLP or allele specific PCR (Hickman, D. *et al.* (1998); Gut 42:402-409). The PCR-RFLP methodology requires the amplification of the fragment of gene containing the A560G mutation. This is performed with the following primers:

5'-TCCTAGAAGACAGCAACGACC-3' (SEQ ID NO:3)

5'-GTGAAGCCCACCAAACAG-3' (SEQ ID NO:4)

This PCR amplification produces a 175 bp fragment that is incubated with the *Bsa*I restriction enzyme. The Nat1*4 allele is cleaved and produces a 155 bp fragment and a 20 bp fragment, while the mutant NAT1*14 is uncleaved.

The NAT1*14 allele is confirmed using an allele specific PCR, with the following primers:

5'-TCCTAGAAGACAGCAACGACC-3' (SEQ ID NO:3)

5'-GGCCATCTTTAAAATACATTTT-3' (SEQ ID NO:5)

CYP2A6

CYP2A6 constitutes 4% of the total CYP 450 enzymes in the human liver. CYP2A6 is estimated as participating in 2.5% of drug metabolism.

POLYMORPHISM

CYP2A6 is functionally polymorphic with two mutant alleles, CYP2A6*2 and CYP2A6*3, resulting in an inactive enzyme or the absence of the enzyme, respectively. Two metabolic phenotypes can be distinguished: poor and extensive metabolizers. CYP2A6 metabolizes several drugs including neuroleptic drugs and volatile anesthetics as well as the natural compounds, coumarin, nicotine and aflatoxin B1.

In addition, CYP2A6 activates several components of tobacco smoke (e.g. NNK), as well as 6-aminochrysene. The role of activation of tobacco smoke and the metabolism of nicotine have suggested a role for CYP2A6 in the development of smoking related cancers.

INDUCTION AND INHIBITION

CYP2A6 is induced by barbiturates, antiepileptic drugs and corticosteroids.

INTER ETHNIC DIFFERENCES

CYP2A6 demonstrates marked inter-individual variability and has demonstrated ethnic related

differences. The proportion of the two phenotypes varied between ethnic groups and countries: % of wt genotype (extensive metabolizers): 85, 76, 52, 83, 97.5 in Finnish, English, Japanese, Taiwanese and African-American populations, respectively. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

NICOTINE

An example of the need for phenotyping in drug dosing is in the delivery of nicotine, for a smoking cessation program. CYP2A6 is the primary means of nicotine metabolism. Extensive CYP2A6 metabolizers will eliminate nicotine at a much higher rate. Identification of individuals with an increased CYP2A6 activity and hence increased nicotine metabolism may identify those individuals that will require higher doses of nicotine at the onset of their attempt to quit smoking with the assistance of a nicotine delivery system. Alternatively, these individuals may benefit from non-nicotine delivery systems for assisting in quitting smoking.

DIRECT PHENOTYPIC DETERMINANTS OF CYP2A6

A probe substrate can be used to determine the CYP2A6 phenotype (coumarin). In accordance with the present invention suitable probe substrates include,

without limitation, coumarin. The structure of coumarin and its metabolite 7-hydroxycoumarin are illustrated in Fig. 5.

In accordance with the present invention, the molar ratio of coumarin and its metabolite, 7-hydroxycoumarin is used to determine the CYP2A6 phenotype of the individual as follows:

$$\frac{\text{7-hydroxycoumarin}}{\text{coumarin}}$$

INDIRECT PHENOTYPIC DETERMINANTS OF CYP2A6 (GENOTYPING)

Currently three alleles have been identified for the CYP2A6 gene, the wild type allele (CYP2A6*1) and two mutant alleles (CYP2A6*2, and CYP2A6*3). The wt allele codes for a fully functional enzyme. The CYP2A6*2 mutant allele codes for an inactive enzyme and the CYP2A6*3 allele does not produce any enzyme.

Determination of an individual genotype can be performed by a combined LA-PCR and PCR-RFLP procedure. In this procedure, specific oligonucleotide primers were used to amplify the CYP2A6/7 gene. The amplified CYP2A6/7 gene is then used as the PCR template to amplify exons 3 and 4 using specific oligonucleotide primers to amplify a 544 bp fragment. This fragment is then digested with the *Fsp*I restriction enzyme and a 489 bp fragment re-isolated. This 489 bp fragment is then incubated with both *Dde*I and *Xcm*I. The digestion patterns were determined by electrophoresis. The wildtype allele produces 330, 87 and

72 bp fragments, the CYP2A6*2 allele yields 189, 141, 87 and 72 bp fragments and the CYP2A6*3 allele yields 270, 87, 72, 60 bp fragments (Nakajima *et al.* (2000) Clin Pharmacol & Ther. 67(1):57-69).

PRIMERS

CYP2A6/7 LA-PCR

5'-CCTCCCTTGCTGGCTGTGTCCCAAGCTAGGC-3'(SEQ ID NO:6)

5'-CGCCCCTTCCTTTCCGCCATCCTGCCCCCAG-3' (SEQ ID NO:7)

EXON 3/4 PCR

5'-GCGTGGTATTCAGCAACGGG-3' (SEQ ID NO:8)

5'-TGCCCCGTGGAGGTTGACG-3' (SEQ ID NO:9)

CYP2C19

CYP2C19 accounts for about 2% of oxidative drug metabolism. CYP2C19 has been postulated as participating in ~8% of drug metabolism.

POLYMORPHISM

Individuals are genetically polymorphic with respect to CYP2C19 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers. Two genetic polymorphisms have been identified (CYP2C19*2 and CYP2C19*3) that together explain all of the Oriental

poor metabolizers and about 83% of Caucasian poor metabolizers. Both of these mutations introduce stop codons resulting in a truncated and non-functional enzyme.

CYP2C19 metabolizes a variety of compounds including the tricyclic antidepressants amitriptyline, imipramine and clomipramine, the sedatives diazepam and hexobarbital, the gastric proton pump inhibitors, omeprazole, pantoprazole, and lansoprazole, as well as the antiviral nelfinavir mesylate, the antimalarial drug proguanil and the β-blocker propanolol.

INDUCTION AND INHIBITION

CYP2C19 is inhibited by fluconazole, fluvoxamine, fluoxetine, sertraline, and ritonavir. It is induced by rifampin.

INTER ETHNIC DIFFERENCES

The occurrence of the poor metabolizer phenotype for CYP2C19 shows a large inter ethnic variability. Poor metabolizers make up less than 4% of the European and white American populations. While the Korean population has a poor metabolizer frequency of 12.6%, the Chinese 17.4% and the Japanese 22.5%. In addition, the CYP2C19 mutant alleles demonstrate interethnic variability with CYP2C19*2 frequency ranging from 28.9% in the Chinese population to only 13% in European-American population. The CYP2C19*3 allele is absent from the European-American or African-American populations, while occurring at a

frequency of 11.7% in both the Korean and Japanese populations.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

OMEPRAZOLE

As an example, the benefit of CYP2C19 metabolic phenotyping in drug dosing is evident in the case of omeprazole. Omeprazole is a drug used in the treatment of *Heliobacter pylori* (*H pylori*) infections in conjunction with amoxicillin, and is cleared from the body via a CYP2C19 metabolic pathway. Studies have observed higher eradication rates of in CYP2C19 poor metabolizers. Therefore, extensive metabolizers may require higher doses of omeprazole to achieve the same level of *H pylori* eradication observed in poor metabolizers. For these reasons, the utility of a reliable phenotyping test for CYP2C19 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective treatment regimes for individuals on an individual basis.

DIRECT PHENOTYPIC DETERMINANTS OF CYP2C19

In accordance with an embodiment of the present invention, the ratio of S-(+)mephenytoin and R-(-)mephenytoin in an urine sample may be used to provide a

determination of an individual's CYP2C19 phenotype. These metabolites are used as quantitative markers in the determination of a CYP2C19 phenotype on the basis of the use of the preferred probe substrate mephenytoin. However, it is fully contemplated that the present invention is not limited in any respect thereto. The structure of R-(-) and S-(+) mephenytoin and 4-hydroxymephenytoin are illustrated in Fig. 6.

The chiral ratio of S-(+)mephenytoin and R-(-)mephenytoin metabolites, used to determine the CYP2C19 phenotype of the individual, is as follows:

$$\frac{\text{S-(+)Mephenytoin}}{\text{R-(-)Mephenytoin}}$$

Chiral ratios of close to unity (>0.8) are indicative of fast CYP2C19 metabolizers.

INDIRECT PHENOTYPIC DETERMINANTS OF CYP2C19 (GENOTYPING)

As mentioned previously the CYP2C19 has two predominant variant alleles, which account for all Japanese poor metabolizers and 83% of Caucasian poor metabolizers. Studies have demonstrated an excellent correlation between a homozygous presence of mutant alleles and poor metabolizer status. An example of a procedure for genotyping CYP2C19 involves a series of polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the

functional CYP2C19*1 allele (Furuta *et al.* (1999) Clin Pharmacol Thera 65(5):552-561; Tanigawara *et al.* (1999) Clin Pharmacol Thera 66(5):528-5534). PCR amplification of exon 5 or exon 4 for CYP2C19*2 and CYP2C19*3 respectively are performed using the following primers:

CYP2C19*2 EXON 5 PRIMERS

5'-AATTACAACCAGAGCTTGGC-3' (SEQ ID NO :10)

5'-TATCACTTTCCATAAAAGCAAG-3' (SEQ ID NO :11)

CYP2C19*3 EXON 4 PRIMERS

5'-AACATCAGGATTGTAAGCAC-3' (SEQ ID NO :12)

5'-TCAGGGCTTGGTCAATATAG-3' (SEQ ID NO :13)

The presence of the G681A mutation in CYP2C19*2 is then detected by digestion with the *Sma*I restriction enzyme. The wild type allele will produce a 120 and a 49 bp fragment, while the CYP2C19*2 allele will remain uncleaved. The CYP2C19*3 allele is detected by incubating the exon 4 PCR product with *Bam*HI. The wild type allele will produce a 233 bp and a 96 bp fragment while the CYP2C19*3 allele will remain uncleaved.

Extensive metabolizing phenotype is assigned to those individuals with at least one allele encoding a functional enzyme. The poor metabolizing phenotype is assigned to individuals lacking two or more functional CYP2C19 alleles.

CYP2C9

The CYP2C9 family of metabolic enzymes accounts for approximately 8% of the metabolic enzymes in the liver. CYP2C9 has been postulated as participating in approximately 15% of drug metabolism.

POLYMORPHISM

Individuals are genetically polymorphic with respect to CYP2C9 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers. Three genetic polymorphisms have been definitively identified, one wild type (CYP2C9*1) and two mutant (CYP2C9*2 and CYP2C9*3). The CYP2C9*2 allele was found to result in 5-10 fold increase in expression of mRNA and have 3-fold higher enzyme activity for metabolism of phenytoin and tolbutamide. Conversely, this genotype appears to have a lower level of activity for the metabolism of S-warfarin. The CYP2C9*3 allele appears to demonstrate decreased metabolic activity against all three of these substrates.

CYP2C9 metabolizes a variety of compounds including S-warfarin, phenytoin, tolbutamide, tienilic acid, and a number of nonsteroidal antiinflammatory drugs such as diclofenac, piroxicam, tenoxicam, ibuprofen, and acetylsalicylic acid.

INDUCTION AND INHIBITION

CYP2C9 is inhibited by fluconazole, metronidazole, miconazole, ketoconazole, itaconazole, ritonavir, clopidrogel, amiodarone, fluvoxamine, sulfamthoxoazole, fluvastatin and fluoxetine. It is induced by rifampin and rifabutin.

INTER ETHNIC DIFFERENCES

The CYP2C9 genotypes demonstrate marked inter ethnic variability. The CYP2C9*2 is absent from Chinese and Taiwanese populations and present in only 1% of African American populations, but accounts for 19.2% of the British population and 8% of Caucasians. CYP2C9*3 is rarer and is present in 6% of Caucasian, 2% of Chinese, 2.6% of Taiwanese and 0.5% of African-American populations.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

S-WARFARIN

As an example, the benefit of CYP2C9 metabolic phenotyping in drug dosing is evident in the case of S-warfarin. S-warfarin is an anticoagulant drug. Studies have demonstrated that the presence of either CYP2C*2 or CYP2C9*3 haplotypes results in a decrease in the dose necessary to acquire target anticoagulation intensity. In

addition, these individuals also suffered from an increased incidence of bleeding complications. Therefore, the CYP2C9 gene variants modulate the anticoagulant effect of the dose of warfarin prescribed. For these reasons, the utility of a reliable test for CYP2C9 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective treatment regimes for individuals on an individual basis.

DIRECT PHENOTYPIC DETERMINANTS OF CYP2C9

In accordance with an embodiment of the present invention, the ratio of Losartan and its metabolite EXP3174 in a urine sample may be used to provide a determination of an individual's CYP2C9 phenotype. These metabolites are used as quantitative markers in the determination of a CYP2C9 phenotype on the basis of the use of the preferred probe substrate Losartan. The structures of Losartan and its metabolite EXP3174 are illustrated in Fig. 7. However, it is fully contemplated that the present invention is not limited in any respect thereto. In fact, due to the nature of the substrate specific alterations caused by the individual CYP2C9 mutations, multiple probe substrates may be necessary for a completely informative phenotypic determination of CYP2C9.

The molar ratio of Losartan and its metabolite, EXP3174 used to determine the CYP2C9 phenotype of the individual, is as follows:

Losartan

EXP3174

INDIRECT PHENOTYPIC DETERMINANTS OF CYP2C9 (GENOTYPING)

As mentioned previously the CYP2C9 has two predominant variant alleles, CYP2C9*2 and CYP2C9*3. An example of a procedure for genotyping CYP2C9 involves a series of polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2C9*1 allele (Taube *et al.* (2000) Blood 96(5):1816-1819). PCR amplification of exon 3 for CYP2C9*2 is performed using the following primers:

CYP2C9*2 EXON 3 PRIMERS

5'-CAATGGAAAGAAATGGAAGGAGGT-3' (SEQ ID NO:14)

5'-AGAAAGTAATACTCAGACCAATCG-3' (SEQ ID NO:15)

A forced mismatch was included in the penultimate base of the forward primer to create a restriction site for the *Ava*II digestion. The PCR product from this amplification is 251 bp in length. After *Ava*II digestion the CYP2C9*1 (wt) allele produces 170 and 60 bp fragments. The CYP2C*2 allele produces a 229 bp fragment.

The CYP2C9*3 allele does not naturally destroy or produce a restriction site. Therefore, a restriction site was introduced into the forward primer such that the

adenosine at position 1061 (A1061) in combination with the mismatch creates a restriction site for the *Nsi*I restriction enzyme. Therefore the PCR amplified fragment of the CYP2C9*1 (wt) allele would have a restriction site at A1061. Conversely, the mutation of A1061C in CYP2C9*3 removes this restriction site. The forward primer also includes a natural *Ava*II restriction sequence. The reverse primer also has a forced mismatch at 1186 to provide a restriction site for the *Nsi*I restriction enzyme (PCR amplified fragments from both the CYP2C9*1 and CYP2C9*3 alleles will have this restriction site). The PCR product for this set of primers prior to restriction enzyme digest is 160 bp in length. Following restriction digest with *Nsi*I and *Ava*II, the CYP2C9*1 allele produces a 130 bp fragment and the CYP2C9*3 allele produces a 140 bp fragment.

CYP2C9*3 PRIMERS

5'-TGCACGAGGTCCAGAGATGC-3' (SEQ ID NO:16)

5'-AGCTTCAGGGTTTACGTATCATAGTAA-3' (SEQ ID NO:17)

Due to the substrate specific alterations in enzyme activity resulting from the two allelic variants, the phenotypic determination will be correlated on an individual substrate basis.

CYP2D6

CYP2D6 constitutes 1-3% of the total CYP 450 enzymes in the human liver. CYP2D6 has been postulated as participating in ~20% of drug metabolism.

POLYMORPHISM

CYP2D6 was the first P450 enzyme to demonstrate polymorphic expression in humans. Three metabolic phenotypes can be distinguished: poor, (PM), extensive (EM) and ultraextensive (UEM) phenotypes. The CYP2D6 gene is extensively polymorphic. For example, a 1997 study documented 48 mutations and 53 alleles of the CYP2D6 gene in a screen of 672 unrelated individuals. Examples of alleles with normal (extensive), wild-type function are CYP2D6*1, CYP2D6*2A, and CYP2D6*2B; alleles resulting in an absence of function are CYP2D6*3, CYP2D6*4A, CYP2D6*4B, CYP2D6*5, CYP2D6*6A, CYP2D6*6B, CYP2D6*7, CYP2D6*8, CYP2D6*11 and CYP2D6*12; and alleles resulting in a reduced function are CYP2D6*9, CYP2D6*10A, and CYP2D6*10B. The ultraextensive phenotype appears to arise from the presence of multiple copies of the CYP2D6 gene (for example, one individual was identified with 13 copies of the gene).

CYP2D6 metabolizes a large variety of drugs and dietary constituents including, but not limited to the following:

ANTIVIRAL AGENTS:

Efavirenz, nevirapine, ritonavir, saquinovir, nelfinavir mesylate, and indinavir

PSYCHOTROPIC DRUGS:

amiflamine, amitryptyline, clomipramine, clozapine, desipramine, haloperidol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphenazine, remoxipride, thioridazine, tomoxetine, trifluperidol, zuclopenthixol, risperidone, and fluoxetine.

CARDIOVASCULAR AGENTS:

aprindine, bufuralol, debrisoquine, encainide, flecainide, guanoxan, indoramin, metoprolol, mexiletin, n-propylamaline, propafenone, propranolol, sparteine, timolol, and verapamil.

MISCELLANEOUS AGENTS:

chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, and phenformin.

In addition, CYP2D6 is involved in the metabolism of many carcinogens, however, as yet it is not reported as the major metabolizer for any. In one study it has been shown that individuals who are fast CYP2D6 metabolizers

and slow N-acetylators are at a greater risk for hepatocellular cancer (OR=2.6; 95% CI=1.6-4).

INDUCTION AND INHIBITION

CYP2D6 is inhibited *in vitro* by quinidine and by viral protease inhibitors as well as by appetite suppressant drugs such as D- and L-fenfluramine.

INTER ETHNIC DIFFERENCES

The activity of CYP2D6 varies broadly in a given population. Poor (PM), extensive (EM) and ultraextensive (UEM) phenotypes of CYP2D6 have been distinguished. The CYP2D6 gene is inherited as an autosomal recessive trait and separates 90 and 10% of the white European and North American population into extensive (EM) and poor (PM) metabolizer phenotypes, respectively. In another study the percentage of PM in different ethnic populations was observed, and white North Americans and Europeans were found to have 5-10% PM's, African-American, 1.8%, Native Thais, 1.2%, Chinese 1%, and Native Malay populations, 2.1%, while the PM phenotype appears to be completely absent in the Japanese population. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

DEXTROMETHORPHAN/ANTIDEPRESSANTS

An example of the need for phenotyping in drug dosing is the case of dextromethorphan. Dextromethorphan is a nonopioid antitussive with psychotropic effects. However, dextromethorphan doses range from 0 to 6 mg/kg based on individual tolerance. Dextromethorphan is activated via the CYP2D6 metabolizing system. Dextromethorphan produced qualitatively and quantitatively different objective and individualive effects in poor vs. extensive metabolizers (mean performance +/-SE, 95+/-0.5% for EMs vs. 86+/-6% for PMs; $p<0.05$).Another important class of drugs for CYP2D6 phenotyping is the tricyclic antidepressants. Both the PM and UEM phenotypes of CYP2D6 are at risk of adverse reactions. PM individuals given standard doses of these drugs will develop toxic plasma concentrations, potentially leading to unpleasant side effects including dry mouth, hypotension, sedation, tremor, or in some cases life-threatening cardiotoxicity. Conversely, administration of these drugs to UEM individuals may result in therapeutic failure because plasma concentrations of active drugs at standard doses are far too low. For, these reasons, the utility of a reliable phenotyping test for CYP2D6 is evident.

PHENOTYPIC DETERMINANTS OF CYP2D6

Different probe substrates can be used to determine the CYP2D6 phenotype (dextromethorphan, debrisoquine, bufuralol, antipyrine, theophylline and hexobarbital). In accordance with the present invention,

suitable probe substrates include without limitation, dextromethorphan, debrisoquine, and bufuralol.

Of these dextromethorphan is the preferred probe substrate. The structure of dextromethorphan and its demethylated metabolite dextrorphan are illustrated in Fig. 8.

In accordance with the present invention, the molar ratio of dextromethorphan and its metabolite is used to determine the CYP2D6 phenotype of the individual as follows:

$$\frac{\text{dextromethorphan}}{\text{dextrorphan}}$$

An antimode of 0.30 is used to differentiate between extensive and poor metabolizers whereby an antimode of less than 0.30 indicates an extensive metabolizer and greater than 0.30 indicates a poor metabolizer.

INDIRECT PHENOTYPIC DETERMINANTS OF CYP2D6 (GENOTYPING)

As mentioned previously the CYP2D6 gene is extensively polymorphic with one study identifying 48 mutations and 53 alleles. An example of a procedure for genotyping CYP2D6 involves the amplification of the entire CYP2D6 coding region (5.1kb product) by XL-PCR using specific primers. This product is then used for a series of polymerase chain reaction - restriction fragment length

polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2D6*1 allele (Garcia-Barceló *et al.* (2000) Clinical Chemistry 46(1):18-23). For example, to detect the C188T transition mutation the following primers can be used to first amplify the CYP2D6 gene and then the specific region of the mutation:

FULL CYP2D6 GENE

5'-CCAGAAGGCTTTGCAGGCTTCA-3' (SEQ ID NO:18)

5'-ACTGAGCCCTGGGAGGTAGGTA-3' (SEQ ID NO:19)

C188T MUTATION

5'-CCATTTGGTAGTGAGGCAGGTAT-3' (SEQ ID NO:20)

5'-CACCATCCATGTTTGCTTCTGGT-3' (SEQ ID NO:21)

The presence of the C188T mutation is then detected by digestion with the *Hph*I restriction enzyme.

In general, the most frequent mutations are examined and these correspond to the most frequent alleles and genotypes.

Extensive metabolizing phenotype is assigned to those individuals with at least one allele encoding a functional enzyme. The poor metabolizing phenotype is assigned to individuals lacking two or more functional CYP2D6 alleles.

CYP2E1

CYP2E1 constitutes approximately 5% of the total CYP 450 enzymes in the human liver.

POLYMORPHISM

The CYP2E1 gene has been demonstrated to be polymorphic in the human population. Studies have demonstrated the presence of 10 CYP2E1 alleles (one wt CYP2E1*1, and 9 mutant, CYP2E1*2, CYP2E1*3, CYP2E1*4, CYP2E1*5A, CYP2E1*5B, CYP2E1*6, CYP2E1*7A, CYP2E1*7B, and CYP2E1*7C). The exact relationship of these polymorphisms to CYP2E1 enzyme activity has not been clarified, however, some studies suggest that the mutant alleles CYP2E1*5A and CYP2E1*5B, result in increased transcription and increased enzyme activity.

CYP2E1 metabolizes several drugs and dietary constituents including isoflurane, halothane, methoxyflurane, enflurane, propofol, thiamylal, sevoflurane, ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, and p-nitrophenol.

In addition, CYP2E1 activates environmental pro-carcinogens especially nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, and 3-hydroxypyridine (tobacco smoke product). In one study it has been shown that individuals who have high CYP2E1 (CYP2E1*5A or CYP2E1*5B) activity are at a greater risk for gastric cancer (OR=23.6-25.7).

INDUCTION AND INHIBITION

CYP2E1 is induced by a number of drugs and environmental factors such as cigarette smoke as well as by starvation, chronic alcohol consumption and in uncontrolled diabetes. CYP2E1 is inhibited by chlormethiazole, trans-1,2-dichloroethylene, disulferan (cimetidine) and by the isoflavonoids genistein and equol.

Induction or inhibition by environmental factors can severely alter an individual's capacity to metabolize certain drugs. Therefore, the present invention may find further application in the individualization of therapy whereby environmental factors are determined to effect an individual's metabolism specific to an enzyme and/or metabolic pathway of interest with respect to a given drug, such as CYP2E1, for example. Furthermore, as environmental factors vary on an individual basis and over time, the present invention may be employed to detect changes in an individual's metabolism specific to an enzyme and/or metabolic pathway of interest due to environmental factors at any given time, and provide valuable phenotype-specific information in the determination of a safe and efficacious individualized treatment regime. By employing the present invention on a routine basis, an individual's treatment regime may be modified to account for environmental influences and maximize the effectiveness of treatment.

INTER ETHNIC DIFFERENCES

The proportion of CYP2E1 phenotypes varied between ethnic groups and countries: the frequency of the rare c2 (CYP2E1*5A or CYP2E1*5B) allele is about 4% in Caucasians and 20% in the Japanese and a study of a separate polymorphism described a rare C allele (CYP2E1*5A or CYP2E1*6) that has a frequency of about 10% in Caucasian and 25% in Japanese populations. In one study it was shown that Japanese males had much lower levels of CYP2E1 activity as compared to Caucasian males. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

ACETAMINOPHEN

An example of the need for phenotyping in drug dosing is the case of acetaminophen. Acetaminophen is a widely used painkiller. However, acetaminophen causes hepatotoxicity at low frequency. The hepatotoxicity is due to its transformation via CYP2E1, to a reactive metabolite (N-acetyl-p-benzoquinoneimine) which is capable of binding to nucleophiles. For these reasons, the utility of a reliable phenotyping test for CYP2E1 is evident.

DIRECT PHENOTYPIC DETERMINANTS OF CYP2E1

In accordance with the present invention a suitable probe substrate is, without limitation, chlorzoxazone.

In accordance with the present invention, the molar ratio of chlorzoxazone and its metabolite is used to determine the CYP2E1 phenotype of the individual as follows:

$$\frac{\text{6-hydroxychlorzoxazone}}{\text{chlorzoxazone}}$$

The structures of chlorzoxazone and its metabolite 6-hydroxychlorzoxazone are illustrated in Fig. 9.

INDIRECT PHENOTYPIC DETERMINANTS OF CYP2E1 (GENOTYPING)

As mentioned previously the CYP2E1 gene has multiple polymorphisms. An example of a procedure for genotyping CYP2E1 for the most common mutations, those termed the *Pst*/*Rsa*I and *Dra*I mutations (allows genotyping of CYP2E1*5A, CYP2E1*5B and CYP2E1*6), involves the amplification of a fragment containing either the *Pst*I and *Rsa*I restriction sites or the *Dra*I restriction site using specific primers (Nedelcheva *et al.* (1996) Methods in Enzymology 272:218-225). The amplified product is then incubated with the appropriate restriction enzyme (*Pst*I or *Rsa*I/*Dra*I) and the digestion products separated electrophoretically. From an allele with wt sequence at

the *Pst*I or *Rsa*I site, the 510 bp fragment produced by PCR is cleaved to a 360 bp and a 150 bp fragment. From the mutant allele the 510 bp fragment remains uncleaved. From an allele with the wt sequence at the *Dra*I mutation site, the 370 bp PCR amplified fragment is cleaved to a 240 bp and 130 bp pair of fragments, while the mutant allele is uncleaved.

*PSTI/RSA*I PRIMERS

5'-CCCGTGAGCCAGTCGAGT-3' (SEQ ID NO :22)

5'-ATACAGACCCTCTTCCAC-3' (SEQ ID NO :23)

*DRA*I PRIMERS

5'-AGTCGACATGTGATGGATCCA-3' (SEQ ID NO :24)

5'-GACAGGGTTTCA-TCATGTTGG-3' (SEQ ID NO :25)

The CYP2E1*5A mutant allele contains both the *Rsa*I and the *Dra*I mutations, while the CYP2E1*5B mutant allele contains the *Rsa*I mutation alone. The *Rsa*I mutation has been associated with an increased expression and increased enzyme activity. Therefore, an individual with two copies of either CYP2E1*5 allele could be considered assigned an extensive metabolizing phenotype. Conversely, the CYP2E1*2 mutation has been associated with decreased protein expression and decreased enzyme activity. Therefore, a person homozygous for the CYP2E1*2 allele could be assigned a poor metabolizing phenotype.

CHARACTERIZATION OF MULTIPLE PHENOTYPIC DETERMINANTS

On the basis of the above enzyme-specific metabolic pathways, several approaches to identifying phenotypic determinants thereof have been developed in accordance with the present invention. The characterization of multiple phenotypes offers multiple applications. The determination of an individual's metabolic phenotype for a multitude of cytochrome P450 and N-acetyltransferase metabolic enzymes allows the use of this single profile for multiple applications. If a drug is metabolized by more than one enzyme, the phenotypic status of each of the enzymes may be important for first, determining if the individual can safely ingest a given drug and second, determining the optimal dose for this individual if they are able to take the drug.

For example, in the case of the antineoplastic agent amonafide, it is suggested that CYP1A2 may, in addition to NAT2, play a minor but nonetheless significant role in the metabolism of this drug. Accordingly, it is contemplated that the ability to characterize multiple phenotypic determinants may also play an important role in the individualization of therapy with amonafide on the basis of phenotyping.

In addition, the knowledge of multiple phenotypes will facilitate the comparison of multiple drugs within the same class or genus, where different metabolic enzymes are involved in the metabolism of these drugs. For example, consider an individual requiring a certain class

of drug, of which there are three that are primarily prescribed. If one is metabolized by CYP1A2, one by CYP2D6 and the remaining drug by CYP3A4, and all individuals that are poor metabolizers of these drugs are at risk for toxicity. Then the drug chosen for treating that individual may be determined on the basis of a phenotypic profile of that individual. If for example the individual is a poor metabolizer for CYP2D6 and CYP3A4, then the first drug metabolized by CYP1A2 may be the first drug to consider for treating the individual.

Another advantage to the determination of an individual's metabolic profile for multiple phenotypic determinants is the effect of a drug on the metabolic status of enzymes not primarily involved in its metabolism. For example, a drug may be metabolized by CYP2C9 and inhibit the activity of CYP3A4. If an individual has very low levels of CYP3A4 to begin with then this inhibition may have little effect on that individuals CYP3A4 phenotype. However, if the individual is an extensive CYP3A4 metabolizer this drug may profoundly alter the CYP3A4 metabolic status. This can cause enormous problems in the case of polypharmacy, where an individual may be taking multiple drugs, and the addition of one drug may affect the safety and efficacy of the pre-existing drug treatment(s).

The metabolic phenotype can be determined directly (by measuring enzyme activity) or indirectly (by examining enzymes genetic sequence). In general, for example, for direct phenotyping, a probe substrate or substrates, such

as those exemplified in Table 7 are administered to an individual to be phenotyped. A biological sample, such as a urine sample is subsequently collected from the individual approximately 4 hours after administering the probe substrate(s). The urine sample is analyzed according to a ligand binding assay, such as enzyme-linked immunosorbent assay (ELISA) technology as described hereinbelow, for metabolites corresponding to the probe substrate(s) and the molar ratios of the metabolites calculated to reveal the individual phenotypes.

In general, for example, for indirect phenotyping, a blood sample of an individual is obtained, and the genetic sequence of the enzyme(s) is examined for the presence or absence of specific mutations. A specific probe for a known allelic variation may be used to screen for a specific genotype known to effect an individual's specific enzymatic capacity. The combination of mutations on the two alleles is matched to known genotypes. The phenotype is then inferred for those genotypes whose presence has been correlated to a known phenotype.

LIGAND-BINDING ASSAYS

The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of both the analytical immunoassay in solution and the immunosensor on solid-state interfaces. The underlying fundamental concept of these analytical methods as ligand-binding assays is based on the observation of the products

of the ligand-binding reaction between the target analyte and a highly specific binding reagent.

The development of immunoassay technology is a success story especially for the clinical laboratory and still continues to be a vibrant area of research. Further development and automation will expand the possibilities of immunoassay analysis in the clinical sciences. Besides this, new areas for trace analyses using immunoassay were defined in the last decade: the environmental analysis of trace substances and quality control in the food industry. Since these applications also need a continuous monitoring mode, the idea of an immunosensor as a continuously working heterogeneous immunoassay system, covering these features, was conceived. The immunosensor is now considered as a major development in the immunochemical field. Despite an overwhelming number of papers is this field, there are only a few commercial applications of immunosensors in clinical diagnostics. The reasons are, in part, unresolved fundamental questions relating to immobilization, orientation, and specific properties of the antibodies or antibody-related reagents on the transducer surface. In addition, a key issue is which clinical applications may benefit most from immunosensor devices in the routine medical laboratory. Only if there is consensus on the clinical utility of this new technique can the gap between the high expectations of the developer and reality be closed. Designers of immunosensor devices must be aware of the general and special needs of laboratory medicine from new analytical techniques.

A new analyzer should be simple and "rugged" for the measurement of analytes. Measurements have to be performed precisely and accurately, even under emergency conditions. The analyzer must be fully automated and capable of performing rapid measurements with turnaround times of < 1 h. Additionally, the determination of an analyte should preferably be without sample pretreatment in matrices, such as serum, plasma, urine or cerebrospinal fluid. All parameters determined with a new analyzer must meet the following criteria, which are defined in various guidelines: low imprecision, small lot-to-lot variations, high analytical sensitivity, optimum analytical specificity and accuracy with long calibration stability and low interferences by drugs or normal and pathological sample components.

In the clinical laboratory, a future substitution of immunoassays by immunosensors simply depends on the superiority and versatility of the new methodology. The applicability for point-of-care testing or when they are temporarily implanted into the individual additionally depends on the reliable and accurate analysis of the desired analyte, without drift problems or matrix interferences. Due to the tremendously growing variety of developments, this review is not intended to be comprehensive. Hence, the main focus will be the description and assessment of reported clinical applications of immunosensors. For a more thorough understanding, we refer to several excellent reviews in the last 5 years on technical aspects and the application of immunosensors in various fields. Other related reviews

deal with antibody engineering developments and latest immunoassay technologies.

ANTIBODIES AS BIOAFFINITY INTERFACE FOR BOTH IMMUNOASSAYS AND IMMUNOSENSORS

It should first be clarified that the specificity for the measurement of analytes in all immunosensor systems, as in the case of immunoassays, is dependent on the application of affinity complexation agents (binding molecules). This pivotal feature is shared by both technologies. New developments in protein engineering for immunoglobulins (including antibody fragments, and chimeric antibodies) or in substituting antibodies by alternative binding components (aptamers are one example) or structures (molecular imprinting is one example) will, therefore, be applicable to either technology, if available. In particular, the possibilities in antibody engineering will enable changes in the affinity and fine specificity of antibodies, as well as the expression of fragments as fusion proteins coupled to reporter molecules.

IMMOBILIZATION PROCEDURES FOR ANTIBODIES

Antibodies have to be properly immobilized on the immunosensor surface, which is mostly part of a flow-through cell. The optimum density and adjusted (but not random) orientation of the antibodies are of paramount importance. Due to the different types of sensing

surfaces, this manipulation can have benefits e.g., improvement of the reaction kinetic parameters, but also unfavorable effects (e.g., increased nonspecific binding, partly destroyed paratope). There are four different types of oriented coupling of antibodies: binding to Fc receptors such as protein A or G or recombinant ArG fusion protein on the surface; binding of other binding partners to structures, covalently linked to the Fc part of the antibody, e.g., the biotin residue on the Fc binds to surface-coated streptavidin; coupling to the solid support via an oxidized carbohydrate moiety on the C2 Fc domain; and the binding of Fab or scFv fragments to the surface of the device via a sulfhydryl group in its C-terminal region.

Numerous chemical reactions can be applied to the immobilization onto solid surfaces. Defined linkages between the antibody or its carbohydrate moieties and the solid phase material (silica, silanized silica, Ta- or Ti-oxides, plastics, sepharose, and metal films) are being built by glutaraldehyde, carbodiimide, uccinimide ester, maleinimide, periodate or galactose oxidase. Moreover, photo-immobilization of antibodies using albumin derivatized with aryldiaziridines as photolinker, is applicable. Physiosorption is not recommended due to the local instability of the layer caused by the mechanical stress in the flow-through cell. An exciting new method for antibody immobilization on a quartz surface of a piezoelectric sensor is based on the deposition of an ethylenediamine plasma polymerization film on the quartz crystal. This film is extremely thin and homogeneous,

incorporating amino functions which may be further derivatized and linked to immunoglobulins, resulting in an orientation-controlled and highly reusable sensing surface. Another recent development is the planar-supported phospho-lipid bilayer (SLB), which can be formed on solid supports by vesicle fusion and Langmuir-Blodgett methods. SLBs maintain two-dimensional fluidity and accommodate multivalent binding between surface-bound ligands and receptor molecules in solution.

For noble metal surfaces, such as gold, in particular, in optical immunosensors, self-assembling monolayer (SAM) techniques seem to be first choice. In general, a SAM is built of long-chained (C_{12} and higher) *n*-alkylthiols with derivatized organic functional groups, which are easily linked to the gold film via the thiol groups by a mechanism still not fully understood. The functional groups of the SAM cross-link with the Fc portion of the antibody (one way is via the biotin streptavidin system), whereas the self-organization of the matrix prevents the surface being individualed to nonspecific binding effects. In addition, the covalent coupling of IgG to a short-chain (thioctic or mercaptopropionic acid are two examples) SAM-modified metal surface has been shown to be an effective affinity-based layer for optical immunosensors.

REGENERATION OF ANTIBODY-COATED SENSOR SURFACES

Conventional homogeneous and heterogeneous immunoassays, respectively, work discontinuously. It is

highly desirable, however, that immunosensor devices, applied in clinical diagnostics, are capable of quasi-continuous recording. The repeated use of disposable sensing elements may mimic a pseudocontinuous action, but this is not considered here. In true immunosensors, the analyte/antigen interaction on the sensor-coated surface is reversible. With the given short incubation times in the flow-through device, the reaction between antigen and antibody is far off the equilibrium state. Fast reversibility and high sensitivity are mutually exclusive of each other. Consistently, an adequate analytical sensitivity is only warranted if antibodies with increased affinity $>10^{10}$ M^{-1} or at least with highly improved on-rate are applied.

The regeneration of the binding sites of the antibodies bound to the immunosensor surface needs stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the immobilized antibodies and insidious drift problems.

Besides this, it must be considered that with the short reaction times between the antibodies and soluble analytes in the flow-through system, the cross-reactivities of the antibody applied can be increased. A highly specific recognition of the antigen is a kinetic-controlled process due to the complexity of the conformational changes in the Fab portion of the antibody upon binding of the antigen.

There are different approaches to solve the "antibody regeneration" problem: one approach is to displace the antigenic analyte by a highly concentrated solution of a related antigen with weak affinity to the surface-bound antibody. However, this depends on the availability of a suitable antigenic surrogate. This is not always feasible and is only applicable to small analytes. A second approach is to use the techniques of antibody engineering to improve the chemical stability of antibodies as whole molecules or as Fab fragments. The phage display technique is such a powerful tool. This can be helpful in the selection of antibody fragments with improved stability. Libraries of mutants of single-chain Fv fragments (scFv), comprising the variable regions of the L and H chains, joined by a peptide linker are generated by a combination of site-directed and random mutagenesis. The selection can be carried out under different physical or chemical pressures to produce thermodynamically more stable scFv mutants. An interesting third approach is a pseudo-regenerating procedure for immunosensors. An amperometric sensor is coated with a conducting immunocomposite, formed by a mixture of specific antibody with methacrylate monomer and graphite. After polymerization, the device is ready for use. Repeated measurements became possible if the polymer is polished thoroughly with abrasive paper. These notes do not apply to immunosensors with a competitive configuration, in which antigenic compounds and not antibodies are surface-immobilized.

ALTERNATIVE ANALYTE-BINDING COMPOUNDS FOR IMMUNOSENSOR APPLICATIONS

APTAMERS

Aptamers are single-stranded DNA or RNA oligonucleotide sequences with the capacity to recognize various target molecules with high affinity and specificity. These ligand-binding oligonucleotides mimic properties of antibodies in a variety of diagnostic formats. They are folded into unique overall shapes to form intricate binding furrows for the target structure. Aptamers are identified by an *in vitro selection process* known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers may have advantages over antibodies in the ease of depositing them on sensing surfaces. Moreover, due to the highly reproducible synthetic approach in any quantities, albeit the affinity constants are consistently lower than those of antibodies and the stability of these compounds is still questionable, they may be particularly useful for diagnostic applications in complex biological matrices. *The aptamer-based schemes are still in* their infancy and it is expected that modified nuclease-resistant RNA and DNA aptamers will soon be available for a variety of therapeutic and diagnostic formats. The potential of aptamers for use in biosensors has been outlined in the design of a fiber-optic biosensor using an anti-thrombin DNA aptamer, immobilized on the surface of silica microspheres and distributed into microwells on the distal

tip of the imaging fiber. With this device, the determination of thrombin at low concentration was possible. Exciting new possibilities are evolving by the introduction of signaling aptamers with ligand-dependent changes in signaling characteristics and catalytically active so-called "apta-zymes" which would allow the direct transduction of molecular recognition to catalysis.

ANTICALINS

Lipocalins constitute a family of proteins for storage or transport of hydrophobic and/or chemically sensitive organic compounds. The retinol-binding protein is an example in human physiology. It has been demonstrated that the bilin-binding protein, a member of the lipocalin family and originating from the butterfly *Pieris brassicae*, can be structurally reshaped in order to specifically complex potential antigens, such as digoxigenin, which was given as an example. These binding proteins share a conserved β-barrel, which is made of eight antiparallel β -strands, winding around a central core. At the wider end of the conical structure, these strands are connected in a pairwise manner by four loops that form the ligand binding site. The lipocalin scaffold can be employed for the construction of so-called "anticalins", which provide a promising alternative to recombinant antibody fragments. This is made by individualizing various amino acid residues, distributed across the four loops, to targeted random mutagenesis. It remains to be shown that this class of proteins is applicable in

diagnostic assays and in immunosensors. Critical points that still need to be defined include the synthesis and stability of the anticalins, the magnitude of the affinity constants, and the versatility for being crafted against the large variety of ligands.

MOLECULAR IMPRINTING TECHNIQUES

This is a technique that is based on the preparation of polymeric sorbents which are selectivity predetermined for a particular substance, or group of structural analogs. Functional and cross-linking monomers of plastic materials, such as methacrylics and styrenes, are allowed to interact with a templating ligand to create low-energy interactions. Subsequently, polymerization is induced. During this process, the molecule of interest is entrapped within the polymer either by a noncovalent, self-assembling approach, or by a reversible, covalent approach. After stopping the polymerization, the template molecule is washed out. The resultant imprint of the template is maintained in the rigid polymer and possesses a steric (size, shape) and chemical (special arrangement of complementary functionality) memory for the template. The molecularly imprinted polymer (MIP) can bind the template (= analyte) with a specificity similar to that of the antigen-antibody interaction.

Besides the main applications in solid-phase extraction and chromatography, molecularly imprinted polymers have already been employed as nonbiological alternatives to antibodies in competitive binding assays.

A series of applications for analytes, such as cyclosporin A, atrazine, cortisol, 17b-estradiol, theophylline, diazepam, morphine, and S-propranolol, suggests that molecular imprinting is a promising technique for immunoassays and immunosensors.

IMMUNOASSAY AND IMMUNOSENSOR TECHNOLOGIES

IMMUNOASSAYS

Immunoassays use antibodies or antibody-related reagents for the determination of sample analytes. This analytical tool has experienced an evolutionary history since 1959, when Berson and Yalow first described the radioimmunoassay (RIA) principle. In the RIA, a fixed and limited amount of antibody is reacted with a fixed and limited amount of radiolabeled antigen tracer and a variable concentration of the analyte. The selectivity of the ligand-binding of antibodies allows these biomolecules to be employed in analytical methods that are highly specific even in complex biological matrices, such as blood, plasma, or urine. By combining the selectivity of antibody-analyte interactions with the vast array of antibodies preformed in immunization processes of host animals and the availability of numerous readily detectable labels radioisotopes, enzymatically or electrochemically induced adsorbance or fluorescence or chemi-luminescence, immunoassays can be designed for a wide variety of analytes while with extraordinarily low detection limits.

BIOSENSORS AND IMMUNOSENSORS

A biosensor is an analytical device that integrates a biological element on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. The biological element is a layer of molecules qualified for biorecognition, such as enzymes, receptors, peptides, single-stranded DNA, or even living cells. If antibodies or antibody fragments are applied as a biological element the device is called an immunosensor. Compared to conventional analytical instruments, biosensors are characterized by an integrated structure of these two components. Many devices are connected with a flow-through cell, enabling a flow-injection analysis (FIA) mode of operation. Biosensors combine high analytical specificity with the processing power of modern electronics to achieve highly sensitive detection systems.

There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses mainly enzymes as the biological compound, catalyzing a signaling biochemical reaction. The bioaffinity-based biosensor, designed to monitor the binding event itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody-related substances for biomolecular recognition. In the latter two cases, these biosensors are called immunosensors.

Biosensors are extensively used as diagnostic tools, predominately in point-of-care testing. Probably the most successful commercialization of biosensors today is the *in vitro* near individual measurement of capillary glucose using various hand-held systems with disposable reagent cartridges.

IMMUNOSENSOR PRINCIPLES

The general immunosensor design is depicted in Fig. 11. There are four types of immunosensor detection devices: electrochemical (potentiometric, amperometric or conductometric/capacitative), optical, microgravimetric, and thermometric. All types can either be run as direct nonlabeled or as indirect labeled immunosensors. The direct sensors are able to detect the physical changes during the immune complex formation, whereas the indirect sensors use signal-generating labels which allow more sensitive and versatile detection modes when incorporated into the complex.

There is a great variety of different labels which have been applied in indirect immunosensors. In principle they are the same labels as used in immunoassays. Among the most valuable labels are enzymes such as peroxidase, glucose oxidase, alkaline phosphatase (AP), catalase or luciferase, electroactive compounds such as ferrocene or In^{2+} salts, and a series of fluorescent labels (including rhodamine, fluorescein, Cy5, ruthenium diimine complexes, and phosphorescent porphyrin dyes). In particular, laser-induced fluorometric resonance energy transfer between two

fluorophores offers methodological advantages and can be extended to fiberoptic sensing.

Although indirect immunosensors are highly sensitive due to the analytical characteristics of the label applied, the concept of a direct sensor device is still fascinating and represents a true alternative development to immunoassay systems. Its potential simplicity holds multiple advantages, making immunosensors progressive and future directed.

The present invention will be illustrated using the following examples, which are not to be seen as limiting in any way. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein Such equivalents are intended to be encompassed in the scope of the claims.

ELECTROCHEMICAL SENSORS

POTENTIOMETRIC IMMUNOSENSORS. The Nernst equation provides the fundamental principle of all potentiometric transducers. According to this equation, potential changes are logarithmically proportional to the specific ion activity. Potentiometric transducer electrodes, capable of measuring surface potential alterations at near-zero current flow, are being constructed by applying the following methodologies.

TRANSMEMBRANE POTENTIAL. This transducer principle is based on the accumulation of a potential across a sensing membrane. Ion-selective electrodes (ISE) use ion-selective membranes which generate a charge separation between the sample and the sensor surface. Analogously, antigen or antibody immobilized on the membrane binds the corresponding compound from the solution at the solid-state surface and changes the transmembrane potential.

ELECTRODE POTENTIAL. This transducer is similar to the transmembrane potential sensor. An electrode by itself, however, is the surface for the immunocomplex building, changing the electrode potential in relation to the concentration of the analyte.

FIELD-EFFECT TRANSISTOR (FET). The FET is a semiconductor device used for monitoring of charges at the surface of an electrode, which have been built up on its metal gate between the so-called source and drain electrodes. The surface potential varies with the analyte concentration. The integration of an ISE with FET is realized in the ion-selective field-effect transistor (ISFET). This technique can also be applied to immunosensors.

An advantage of potentiometric sensors is the simplicity of operation, which can be used for automation, and the small size of the solid-state FET sensors. All potentiometric methods, however, are still suffering from major problems of sensitivity, being inferior to amperometric transducers and nonspecific effects of binding or signaling influences from other ions present in

the sample. Especially, the signal-to-noise ratio causes analytical problems, which are difficult to circumvent. Thus, a trend away from these techniques has been observed in the last few years. However, the ISFET may be seen as a candidate for ultrasensitive clinical immunosensor applications, in particular, when the novel concept of differential ISFET-based measurement of the zeta potential is used. The streaming potential is a potential difference in flow direction, caused by the flow of excess ions resulting from a local distortion of the charge balance. The zeta potential, directly correlated to the streaming potential, reflects the potential changes in the diffuse outer layer at the solid-liquid interface. It efficiently reacts to protein accumulations onto sensor surfaces and, thus, is suitable for detecting immunocomplex reactions.

AMPEROMETRIC IMMUNOSENSORS.

Amperometric immunosensors are designed to measure a current flow generated by an electrochemical reaction at constant voltage. There are only few applications available for direct sensing, since most protein analytes are not intrinsically able to act as redox partners in an electrochemical reaction. Therefore, electrochemically active labels directly or as products of an enzymatic reaction are needed for the electrochemical reaction of the analyte at the sensing electrode. Oxygen and H_2O_2 electrodes are the most popular. An oxygen electrode consists of an electrolyte-bearing chamber with a sensing Pt cathode, polarized at 0.7 V, and an Ag/AgCl reference

electrode. The chamber is gas-permeable, covered by an O_2-pervious membrane.

Besides oxygen, generated by catalase from H_2O_2 there are other amperometrically detectable compounds, such as ferrocene derivatives or In^{2+} salts. A novel approach is the use of the redox polymer [PVP-Os(bipyridyl)$_2$Cl), which is coimmobilized with specific antibodies. Additionally, there are examples for enzymes with electrochemically active products. AP, for example, catalyzes the hydrolysis of phenyl phosphate or *p*-aminophenyl phosphate (4-APP) compounds, which result in electrochemically active phenol or *p*-aminophenol. Furthermore, enzymes, such as horseradish peroxidase (HRP), glucose oxidase, glucose-6-phosphate dehydrogenase, with subsequent amperometrical oxidation of NADH and others, have also been successfully applied as labels.

The main disadvantage for amperometric immunosensors of having an indirect sensing system, however, is compensated for by an excellent sensitivity. This is due to a linear analyte concentration range compared to a logarithmic relationship in potentiometric systems. Special attention must be directed to the system-inherent transport rate limitations for redox partners on the electrode surface.

CONDUCTOMETRIC AND CAPACITIVE IMMUNOSENSORS

These immunosensor transducers measure the alteration of the electrical conductivity in a solution at constant voltage, caused by biochemical enzymatic

reactions which specifically generate or consume ions. The capacitance changes are measured using an electrochemical system, in which the bioactive element is immobilized onto a pair of noble metal, mostly Au or Pt, electrodes. There are only few clinical applications available, as the high ionic strength of biological matrices makes it difficult to record the relatively small net conductivity changes caused by the signaling reaction. To circumvent this problem, recently, an ion-channel conductance immunosensor, mimicking biological sensory functions, was developed. The basis of this technique is the fact that the conductance of a population of molecular ion channels, built of tethered gramicidin A and aligned across a lipid bilayer membrane, is changed by the antibody-antigen binding event. Different applications using various antibodies, linked to the ion-channel complex, are given.

Another approach is the measurement of changes of the surface conductivity. For example, a conductometric immunosensor for the determination of methamphetamine (MA) in urine was recently developed. Anti-MA antibodies were immobilized onto the surface of a pair of platinum electrodes. The immunocomplex formation caused a decrease in the conductivity between the electrodes. The measurement of the reciprocal capacitance, performed at alternating voltage, is advantageous compared to conductometric devices, and serves two purposes. The first is to test the insulating monolayer on the sensor noble metal surface. Self-assembling monolayers, have insulating properties. Besides this, they prevent the immunosensor from being affected by nonspecific binding phenomena. Even

minor desorption of the monolayer results in an essential increase in capacitance. Thus, the actual quality of the device can be checked. The second application is the measurement of changes in the effective dielectric thickness of the insulating layer during antigen binding, when antibodies are linked to the alkylthiol layer. Of course, this is on condition that the v-substitution of the alkylthiol monolayer does not compromise the insulation. Hence, a marked decrease of the electrical capacitance is observed and is used to quantitate the analyte. The destructive influence of lateral diffusion on nanostructured monolayers is prevented by use of the spreader-bar technique.

OPTICAL SENSORS

Optical immunosensors are most popular for bioanalysis and are today's largest group of transducers. This is due to the advantages of applying visible radiation compared to other transducer techniques. Additional benefits are the nondestructive operation mode and the rapid signal generation and reading. In particular, the introduction of fiber bundle optics ("optodes") as optical waveguides and sophisticated optoelectronics offers increased versatility of these analytical devices for clinical applications.

Changes in adsorption, fluorescence, luminescence, scatter or refractive index (RI) occur when light is reflected at sensing surfaces. These informations are the

physical basis for optical sensor techniques. Usually, applied detectors are photodiodes or photomultipliers.

There are numerous applications of either direct label-free optical detection of the immunological reaction, of labeled immunospecies, or of the products of enzymatic reactions. Most labels are fluorescent, but bio- and chemiluminescence species are also possible. It is worth mentioning that the label-free evanescence wave-related sensors explicitly represent an elegant methodology, which is a valuable alternative to sophisticated immunoassays. Nevertheless, label-free systems are prone to unsolved problems, such as nonspecific binding effects and poor analytical sensitivity to analytes with low molecular weight. Kubitschko *et al.* noted that despite the efforts, all immunosensors are still one magnitude less sensitive than commercial immunoassays for determining analytes in human serum, particularly those with low molecular weight. They claim the use of mass labels, such as latex particles, in order to enhance the signal. The authors demonstrated the optimization of a nanoparticle enhanced bidiffractive grating coupler immunosensor for the detection of thyroid-stimulating hormone (TSH, MW 28,000 Da). The excellent performance characteristics of this sensor clearly showed how future devices should work. The problem of unspecific binding, however, can also be controlled by applying a reference sensing region on the chip.

TOTAL INTERNAL REFLECTION SPECTROSCOPY (TIRS)

The common principle of the following analytical devices is that in an optical sensor with two materials with different refractive indices (RI), total internal reflection occurs at a certain angle of the light beam being directed through the layer with the higher RI towards the sensing interface. By this, an evanescence wave is generated in the material with the lower RI. This wave, being an electrical vector of the wavelength of the incident light beam, penetrates further into the medium with exponentially attenuated amplitude. Biomolecules attached in that portion of the medium will interact inevitably with the evanescent wave and, therefore, lead to a distinctive diminution of the reflected light. The resolution is directly proportional to the length of interaction. Infrared spectroscopes, measuring attenuated total reflectance, are commonly built in the Kretschmann configuration: an optically absorbing film at the sensor's surface enables the measurement of the attenuated light intensity as a function of the wavelength of the incident beam. For total internal reflection fluorescence (TIRF), analytics benefit from the fact that incident light excites molecules with fluorescence characteristics near the sensor surface creating a fluorescent evanescent wave. The emerging fluorescence is finally detected. The technique has been developed mainly for an optical detection of fluorescence-labeled antibodies or antigens. In the latter case, the fluorescence capillary fill device (FCFD) technique is worth mentioning. The FCFD is designed by using a planar optical waveguide and a glass plate

separated from each other by a capillary gap. Fluorophore-labeled antigen is attached on the surface of the glass plate, whereas antibodies are immobilized on the surface of the optical waveguide.

Another phenomenon, the optical diffraction, is used by the optical biosensor assay (OBA™) system: biomolecules are attached to the surface of a silanized wafer. The protein-coated surface is illuminated through a photo mask to create distinct periodic areas of active and inactive protein. Upon illumination with laser light, the diffraction grating caused by the ligand-binding process diffracts the incident light. An analyte-free negative sample does not result in diffraction because no antigen-antibody binding occurred creating the diffraction grating. The presence or absence of a diffraction signal differentiates between positive and negative samples. The intensity of the signal provides a quantitative measure of the analyte concentration.

ELLIPSOMETRY

If linearly polarized light of known orientation is reflected at oblique incidence from a surface, the reflected light is elliptically polarized. The shape and orientation of the ellipse depend on the angle of incidence, the direction of the polarization of the incident light, and the reflection properties of the surface. On adsorption of biomolecules onto a planar solid surface, phase and amplitude of the reflected light are altered and can be recorded by ellipsometric techniques.

These changes in the polarization of the light are due to the alterations of the RI and the coating thickness. There are only few applications, such as the study of a cholera toxin-ganglioside GM1 receptor-ligand reaction, which were carried out using an ellipsometer.

OPTICAL DIELECTRIC WAVEGUIDES

Optical waveguides are glass, quartz or polymer films or fibers made of high RI material embedded between or in lower index dielectric materials. If a linearly polarized helium-neon laser light wave, introduced into the high index film or fiber, arrives at the boundary at an angle which is greater than the critical angle of total reflection, it is confined inside the waveguide. Similar to surface plasmon resonance, an evanescent field develops at the sensor's surface. In this case, however, the evanescent field is generated by the excitation of the light itself in the dielectric layer. Most of the laser light is transmitted into the device and multiple reflections occur as it travels through the medium if a bioactive substance is placed over the surface. Some of the light, however, penetrates the biolayer. This light is reflected back into the waveguide with a shift in phase interfering with the transmitted light. Thus, changes in properties of the biolayer can be followed by detecting the changes in interference.

Waveguides are often made in the form of fibers. These fiber-optic waveguide systems offer advantages for sensors when being used for hazardous analysis. Planar

waveguide systems are also applicable for interferometers. They use laser light directed towards the surface of the waveguide with the attached biomolecules, which is subsequently split into two partial electrical (TE) and magnetic (TM) fieldwaves, perpendicular to each other. The interaction with the sample surface changes the relative phase between TE and TM by the different RI and surface thickness values. Various configurations, such as the Fabry-Perot monomode channel interferometer, the Mach-Zehnder interferometer or the related two-mode thin-film waveguide difference interferometer, have been successfully established.

Another technique uses thin corrugations etched into the surface of a waveguide. This grating coupler device allows the measurement of the coupling angle of either the input or output laser beam. Both beams are correlated to the RI within the evanescent field at the sensor's surface. Recently, a long-period grating fiber immunosensor has proven to be sensitive (enabling analyses down to the nanomolar range) and reproducible. Grating couplers are also used for optical waveguide lightmode spectroscopy (OWLS). The basic principle of the OWLS method is that linearly polarized light is coupled by a diffraction grating into the waveguide layer. The incoupling is a resonance phenomenon that occurs at a defined angle of incidence that depends on the RI of the medium covering the surface of the waveguide. In the waveguide layer, light is guided by total internal reflection to the edges where it is detected by photodiodes. By varying the angle of incidence of the

light, the mode spectrum is obtained from which the effective RIs are calculated for both TE and TM.

SURFACE PLASMON RESONANCE (SPR)

Among the different detection systems, SPR is the most popular one. There are two leading systems on the market: the BIAcore™ systems from Biacore (Uppsala, Sweden) and the IAsys™ from Fisons Applied Sensor Technology (Cambridge, UK). Other systems with small market positions are the BIOS-1 from Artificial Sensing Instruments (Switzerland), the SPR-20 from Denki Kagaku Keiki (Japan), the SPEETA from Texas Instruments (USA), the IBIS from Windsor Scientific (UK) and the DPX from Quantech (USA). The first two commercial evanescence-wave devices are widespread in research laboratories due to the sophisticated apparatus and userfriendly control software. The BIAcore™, however, has the biggest market position.

The general principle of SPR measurement 80 is depicted in Fig. 12. Polarized light is directed from a layer of high RI towards a layer with low RI to result in total internal reflection. The sample is attached to the layer of low RI. At the interface between the two different media, a thin approximately 50 nm gold film is interposed. Although light does not propagate into the low RI medium, the interfacial intensity is not equal to zero. The physical requirement of continuity across the interface is the reason for exciting the surface electrons "plasmons" in the metal film by the light energy. As a

result, the electrons start oscillating. This produces an exponentially decaying evanescent wave penetrating a defined distance into the low RI medium, which is accountable for a characteristic decrease in the intensity of the reflected light. Hence, a direct insight in changes of the RI at the surface interface is made possible by monitoring the intensity and the resonance angle of the reflected light, caused by the biospecific interactions which took place there. Whereas in the BIAcore™ system, the light affects the sensing layer only once, there are several propagation contacts in the IAsys™ due to the device's resonant mirror configuration. The BIAcore™ SPR apparatus is characterized by a sensitive measurement of changes of the RI when polarized laser light is reflected at the carboxy-methylated dextranactivated device interface. The IAsys™ SPR device also uses a carboxy-methylated dextran-activated surface. Its dextran layer, however, is not attached to a gold surface, but to titanium, which forms a high refractive dielectric resonant layer. The glass prism is not attached tightly on the opposite side of the titanium layer, making space for an interposed silica layer of low RI. By this layer, the laser light beam couples into the resonant layer via the evanescent field. Therefore, the IAsys™ is seen as a combination of SPR resonant mirror with waveguide technology. As a result, no decrease in the reflected light intensity at resonance is observed in this system. The specific signal is the change in the phase of the reflected polarized light.

Differential SPR, a novel modification of a SPR immunosensor, improves further the sensitivity of the sensor by applying a modulation of the angle of light incidence. The reflectance curve is measured with a lock-in amplifier and recorded in the first and second derivative.

Light is directed from a prism with a RI towards a layer with low RI, resulting in total internal reflection. Although light does not propagate into the medium, the interfacial intensity is not equal to zero. Physical requirements of continuity across the interface are the cause of excitation of surface plasmons in the metal film by the light energy, causing them to oscillate. This produces an exponential evanescent decaying, which penetrates a defined distance into the low-index medium and results in a characteristic decrease in reflected light intensity.

MICROGRAVIMETRIC SENSORS

A direct measurement of mass changes induced by the forming of antigen/antibody complexes is also enabled by acoustic sensors. The principle of operation is based on the propagation of acoustic shear waves in the substrate of the sensor. Phase and velocity of the acoustic wave are influenced by the specific adsorption of antibody molecules onto the antigen-coated sensor surface. Piezoelectric materials, such as quartz (SiO_2), zinc oxide (ZnO) or others resonate mechanically at a specific ultrasonic frequency in the order of tens of megahertz

when excited in an oscillating electrical field. The resonant frequency is determined by the distance between the electrodes on both sides of the quartz plate, which is equal to the thickness of the plate and the velocity of the acoustic wave within the quartz material. In other words, electromagnetic energy is converted into acoustic energy, whereby piezoelectricity is associated with the electrical polarization of materials with anisotropic crystal structure. The most applied technique for monitoring the acoustic wave operation is the oscillation method. This means a configuration in which the device constitutes the frequency-controlling element of a circuit. The oscillation method measures the series resonant frequency of the resonating sensor.

The microgravimetric sensor devices are divided into quartz crystal microbalance (QCM) devices applying a thickness-shear mode (TSM), and devices applying a surface acoustic wave (SAW) detection principle. These sensors have reached considerable technical sophistication.

Additional bioanalytical application devices include the flexural plate wave (FPW), the shear horizontal acoustic plate (SH-APM), the surface transverse wave (STW) and the thin-rod acoustic wave (TRAW)

There are considerable similarities between the physical principles of QCM and SPR sensors, even when there are fundamental differences. Both QCM and SPR are wave-propagation phenomena and show resonance structure. The elastic QCM wave and the surface plasmon wave are nonradiative, i.e., an evanescent wave exists. Changes of

physical properties within the evanescent field lead to a shift of resonance. Thus, a linear approximation of the physical relationship is allowed for immunological application in immunosensors.

THE TSM SENSOR

The TSM sensor consists of an AT-cut piezoelectric crystal disc, most commonly of quartz because of its chemical stability in biological fluids and resistance to extreme temperatures. The disc is attached to two metal electrodes on opposite sides for the application of the oscillating electric field. The TSM is run in a range of 5-20 MHz. The schematic design of a typical TSM device shown in Fig. 13. Advantages are, besides the chemical inertness, the low cost of the devices and the reliable quality of the mass-produced quartz discs. Major drawbacks of the system are the insensitivity for analytes with a molecular weight ~1000 Da, and, as seen in all label-free immunosensor systems, nonspecific binding interferences. Nonspecific binding effects are hard to distinguish from authentic binding events due to the fact that no reference line can be placed in the sensor device. For a SH-APM device, however, by appropriately selecting the device frequency, these spurious responses can be suppressed. This sensor is applicable for measurements in human serum matrix.

One of the first applications of TSM technology was an immunosensor for human immunodeficiency virus (HIV) serology. This sensor was realized by immobilizing

recombinant viral peptides on the surface of the transducer and by detecting anti-HIV antibodies directly in human sera.

THE SAW SENSOR

SAW sensors use thick ST-cut quartz discs and interdigitated metal electrode arrays that generate acoustic Rayleigh waves in both directions from the interdigital electrodes, their transmission being attenuated by surface-attached biomolecules. The oscillation frequency of a SAW sensor ranges from 30 to 500 MHz. The operation of SAW immunosensors with biological samples is compromised by the fact that the surface wave is considerably attenuated in the liquid phase. Thus, the domain of this technique is most likely restricted to gas phase operations.

The present invention is exemplified as an ELISA as described hereinbelow for corresponding probe substrate and or metabolites and the molar ratios thereof calculated to reveal the individual phenotypes.

Table 7

Examples of Enzymes and Corresponding Probes Drugs

Enzyme	Probe substrate
NAT1	p-aminosalicylic acid
NAT2	Caffeine
CYP1A2	Caffeine
CYP2A6	Coumarin
CYP2C9	s-ibuprofen
CYP2C19	Mephenytoin
CYP2D6	Dextromethorphan
CYP2E1	Chlorzoxazone
CYP3A4	Midazolam

In Example I, a detailed description of the synthesis of probe substrate and metabolite derivatives and the ELISA development for N-acetyltransferase(NAT2) are illustrated. The materials and methods, and the overall general process described for the development of the NAT2 ELISA method and kit for metabolic are adapted to the development of the metabolic phenotyping ELISA kits for other metabolic enzymes including NAT1, CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19, as well as a multi-determinant metabolic phenotyping system and method. In particular, the protocol as herein described for the development of an ELISA specific to NAT2 is adapted for the development of a CYP3A4-specific ELISA, in accordance with the present invention. Accordingly, an assay system is provided that is adapted for the characterization of phenotypic determinants of CYP3A4 and can be used for individualizing treatment with anticoagulants. Furthermore, the present invention may

also be adapted to provide for the identification of other characteristics or determinants of drug clearance and drug toxicity known to vary on an individual basis.

EXAMPLE I

DETERMINATION OF PHENOTYPIC DETERMINANTS BY ELISA

NAT2

Different probe substrates can be used to determine the NAT2 phenotype (Kilbane, A.J. *et al.* (1990) *Clin. Pharmacol. Ther.*, 47:470-477; Tang, B-K. *et al.* (1991) *Clin. Pharmacol. Ther.*, 49:648-657). In accordance with the present invention caffeine is the preferred probe because it is widely consumed and relatively safe (Kalow, W. *et al.* (1993) *Clin. Pharmacol. Ther.*, 53:503-514). In studies involving this probe, the phenotype has been generally determined from ratios of the caffeine metabolites 5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU) and 1-methylxanthine (1X). In these studies, the individuals are given an oral dose of a caffeine-containing substance, and the urinary concentrations of the target metabolites determined by HPLC (Kilbane, A.J. *et al.* (1990) *Clin. Pharmacol. Ther.*, 47:470-477; Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, 49:648-657) or CE (Lloyd, D. *et al.* (1992) *J. Chrom.*, 578:283-291).

The number of clinical protocols requiring the determination of NAT2 phenotypes is rapidly increasing and

in accordance with the present invention, an enzyme linked immunosorbent assay (ELISA) was developed for use in these studies (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) *J. Pharm. Biomed. Anal.*, 13:1079-1086). ELISAs have been successfully applied in the determination of low amounts of drugs and other antigenic components in plasma and urine samples, involve no extraction steps and are simple to carry out.

In accordance with the present invention, antibodies were raised in animals against two caffeine metabolites [5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU), and 1-methyl xanthine (1X)] present in urine samples of an individual collected after drinking coffee. Their ratio provides a determination of an individual's N-acetylation (NAT2) phenotype. Subsequently, there was developed a competitive antigen enzyme linked immunosorbent assay (ELISA) for measuring this ratio using these antibodies.

The antibodies of the present invention can be either polyclonal antibodies or monoclonal antibodies raised against two different metabolites of caffeine, which allow the measurement of the molar ratio of these metabolites.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the acetylation phenotype of the individual as follows. Individuals with a ratio less than 1.80 are slow acetylators.

MATERIALS AND METHODS

MATERIALS

Cyanomethylester, isobutyl chloroformate, dimethylsulfate, sodium methoxide, 95% pure, and tributylamine were purchased from Aldrich (Milwaukee, WI, USA); horse radish peroxidase was purchased from Boehringer Mannheim (Montreal, Que., Canada); Corning easy wash polystyrene microtiter plates were bought from Canlab (Montreal, Que., Canada); o-methylisourea hydrochloride was obtained from Lancaster Laboratories (Windham, NH, USA); alkaline phosphatase conjugated to goat anti-rabbit IgGs was from Pierce Chemical Co. (Rockford, IL, USA); bovine serum albumin fraction V initial fractionation by cold alcohol precipitation (BSA), complete and incomplete Freund's adjuvants, diethanolamine, 1-methylxanthine, p-nitrophenol phosphate disodium salt, o-phenylenediamine hydrochloride; porcine skin gelatin, rabbit serum albumin (RSA); Sephadex™ G25 fine, Tween™ 20 and ligands used for testing antibodies' cross reactivities were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Whatman™ DE52 diethylaminoethyl-cellulose was obtained from Chromatographic Specialties Inc. (Brockville, Ont., Canada). Dioxane was obtained from A&C American Chemicals Ltd. (Montreal, Que., Canada) and was refluxed over calcium hydride for 4 hours and distilled before use. Other reagents used were of analytical grade.

SYNTHETIC PROCEDURES

The synthetic route for the production of AAMU-hemisuccinic acid (VIII) and 1-methylxanthine-8-propionic acid (IX) is presented in Fig. 14.

SYNTHESIS OF 2-METHOXY-4-IMINO-6-OXO-DIHYDROPYRIDINE (III)

Compound III is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-2276) as follows. To a 250 mL round bottom flask 12.2 g of o-methylisourea hydrochloride (110.6 mmol), 11.81 mL methylcyanoacetate (134 mmol), 12.45 g of sodium methoxide (230.5 mmol) and 80 mL of methanol are added. The suspension is stirred and refluxed for 5 hours at 68-70°C. After cooling at room temperature, the suspension is filtered through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL), and the NaCl on the filter is washed with methanol. The filtrate is filtered by gravity through a Whatman™ no. 1 paper in a 500 mL round bottom flask, and the solvent is evaporated under reduced pressure with a rotary evaporator at 50°C. The residue is solubilized with warm distilled water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered under vacuum through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL). The product is washed with water, acetone, and dried. The product is recrystallized with water as the solvent and using charcoal for

decolorizing (activated carbon, Norit[r] A< 100 mesh, decolorizing). The yield is 76 %.

SYNTHESIS OF 1-METHYL-2-METHOXY-4-IMINO-6-OXO-DYHYDROPYRIMIDINE (IV)

Compound IV is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-2276) as follows. To a 250 mL round bottom flask 11g of compound III (77.0 mmol) and 117 mL of 1N NaOH (freshly prepared) are added. The solution is stirred and cooled at 15°C, using a water bath and crushed ice. Then 11.7 mL dimethylsulfate (123.6 mmol) are added dropwise with a pasteur pipette over a period of 60 min. Precipitation eventually occurs upon the addition. The suspension is stirred at 15°C for 3 hours and is left at 4° C overnight. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL). The yield is 38 %.

SYNTHESIS OF 1-METHYL-4-IMINOURACIL (V)

Compound V is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-2276) as follows. To a 250 mL round bottom flask 11.26 g of compound IV (72.6 mmol) and 138 mL 12 N HCl are added, and the suspension is stirred at room temperature for 16-20 hours. The suspension is cooled on crushed ice, the product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 40-60 ASTM, 60

mL). The product is washed with water at 4°C, using a pasteur pipette, until the pH of filtrate is around 4 (about 150 mL). The product is washed with acetone and dried. The yield is 73 %.

SYNTHESIS OF 1-METHYL-4-IMINO-5-NITROURACIL (VI)

Compound VI is synthesized according to the procedure of Lespagnol et al (Lespagnol, A. et al.(1970) *Chim. Ther.*, 5:321-326) as follows. To a 250 mL round bottom flask 6.5 g of compound V (46 mmol) and 70 mL of water are added. The suspension is stirred and refluxed at 100°C. A solution of 6.5 g sodium nitrite (93.6 mmol) dissolved in 10 mL water is added gradually to the reaction mixture with a pasteur pipette. Then 48 mL of glacial acetic acid is added with a pasteur pipette. Upon addition, precipitation occurs and the suspension becomes purple. The suspension is stirred and heated for an additional 5 min., and cooled at room temperature and then on crushed ice. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 10-15 ASTM, 60 mL). It is washed with water at 4 °C to remove acetic acid and then with acetone. Last traces of acetic acid and acetone are removed under a high vacuum. The yield is 59 %.

SYNTHESIS OF 1-METHYL-4,5-DIAMINOURACIL (VII)

Compound VII is synthesized by the procedure of Lespagnol *et al.* (Lespagnol, A. *et al.*(1970) *Chim. Ther.*,

5:321-326) as follows. To a 100 mL round bottom flask 2 g of compound VI (11.7 mmol) and 25 mL water are added. The suspension is stirred and heated in an oil bath at 60°C. Sodium hydrosulfite (88%) is gradually added (40.4 mmol), using a spatula, until the purple color disappears (approximately 5 g or 24.3 mmol). The suspension is heated for an additional 15 min. The suspension is cooled on crushed ice and left at 4°C overnight. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 30-40 ASTM, 15 mL). The product is washed with water and acetone, and dried. The last traces of acetone are removed under a high vacuum. The yield is 59%.

SYNTHESIS OF AAMU-HEMISUCCINIC ACID (VIII)

Compound VIII is synthesized as follows. To a 20 mL beaker 0.30 g of compound VII (1.92 mmol) and 5 mL water are added. The suspension is stirred and the pH is adjusted between 8 to 9 with a 3N NaOH solution. Then 0.33 g succinic anhydride (3.3 mmol) is added to the resulting solution, and the mixture is stirred until the succinic anhydride is dissolved. During this process, the pH of the solution is maintained between 8 and 9. The reaction is completed when all the succinic anhydride is dissolved and the pH remains above 8. The hemisuccinate is precipitated by acidification to pH 0.5 with 12N HCl. The product is recovered by filtration on a Whatman™ No. 1 paper, and washed with water to remove HCl. It is then washed with acetone and dried.

OTHER AAMU OR AFMU DERIVATIVES

The derivatives shown in Figs. 15 and 16 can also be used for raising antibodies against AAMU or AFMU that can be used for measuring the concentrations of these caffeine metabolites in urine samples.

SYNTHESIS OF 1-METHYLXANTHINE-8-PROPIONIC ACID (IX)

This product is synthesized according to a modified procedure of Lespagnol et al. (Lespagnol, A. et al.(1970) *Chim. Ther.*, 5:321-326) as follows. A 0.2 g sample of compound VIII (0.78 mmol) is dissolved in 2-3 mL of a 15% NaOH solution. The resulting solution is stirred at 100°C until all of the solvent is evaporated, and is then maintained at this temperature for an additional 5 min. The resulting solid is cooled at room temperature, and dissolved in 10 mL water. The product is precipitated by acidification to pH 2.8 with 12 N HCl. After cooling at 4°C for 2.5 hours, the product is recovered by filtration on a Whatman™ No. 1 paper, washed with water and acetone, and dried. It is recrystallized from water-methanol (20:80, v/v), using charcoal to decolorize the solution.

OTHER DERIVATIVES OF 1X

The other derivatives of 1X, shown in Figs. 17 and 18, can also be used for raising antibodies against 1X and thereby to allow the development of an ELISA for measuring 1X concentration in urine samples.

SYNTHESIS OF AAMU

AAMU is synthesized from compound VII according to the procedure of Fink et al (Fink, K. et al. (1964) *J. Biol. Chem.*, 249:4250-4256) as follows. To a 100 mL round bottom flask 1.08 g of compound VII (6.9 mmol) and 20 mL acetic acid anhydride were added. The suspension is stirred and refluxed a 160-165 ^{o}C for 6 min. After cooling at room temperature, the suspension is filtered under vacuum through a sintered glass funnel (Pyrex, 10-15 ASTM, 15 mL). The product is washed with water and acetone, and dried. The product is recrystallized in water.

NMR SPECTROSCOPY

^{1}H and ^{13}C NMR spectra of compounds VIII and IX are obtained using a 500 MHz spectrophotometer (Varian™ XL 500 MHz, Varian Analytical Instruments, San Fernando, CA, USA) using deuterated dimethyl sulfoxide as solvent.

CONJUGATION OF HAPTENS TO BOVINE SERUM ALBUMIN AND RABBIT SERUM ALBUMIN

The AAMU-hemisuccinic acid (VIII) and the 1-methylxanthine propionic acid (IX) are conjugated to BSA and RSA according to the following mixed anhydride method. To a 5 mL round bottom flask 31.7 mg of compound VIII (0.12 mmol) or 14.9 mg of compound IX (0.06 mmol) are added. Then 52.2 µL of tri-n-butylamine (0.24 mmol) and

900 µL of dioxane, dried over calcium hydride and freshly distilled, are added. The solution is cooled at 10°C in a water bath using crushed ice. Then 12.6 µL isobutyl chloroformate at 4°C (0.12 mmol, recently purchased or opened) are added and the solution is stirred for 30-40 min at 10-12°C. While the above solution is stirring, a second solution is prepared as follows. In a glass tube 70 mg BSA or RSA (0.001 mmol) are dissolved in 1.83 mL water. Then 1.23 mL dioxane, freshly dried and distilled, is added and the BSA or RSA solution is cooled on ice. After 30-40 min of the above stirring, 70 µL of 1 N NaOH solution cooled on ice is added to the BSA or RSA solution and the resulting solution is poured in one portion to the flask containing the first solution. The solution is stirred at 10-12°C for 3 hours and dialyzed against 1 liter of water for 2 days at room temperature, with water changed twice a day. The protein concentration of the conjugates and the amounts of moles of AAMU or 1X incorporated per mole of BSA or RSA is determined by methods described below. The products are stored as 1 mL aliquots at -20°C.

PROTEIN DETERMINATION BY THE METHOD OF LOWRY ET AL. (Lowry, O.H. et al. (1951) *J. Biol. Chem.*, 193:265-275)

A) SOLUTIONS

Solution A: 2 g Na_2CO_3 is dissolved in 50 mL water, 10 mL of 10 % SDS and 10 mL 1 NaOH, water is added to 100 mL. Freshly prepared.

Solution B: 1 % NaK Tartrate

Solution C: 1 % $CuSO_4.5H_2O$

Solution D: 1 N phenol (freshly prepared): 3 mL Folin & Ciocalteu's phenol reagent (2.0 N) and 3 mL water.

Solution F: 98 mL Solution A, 1 mL Solution B, 1 mL Solution C. Freshly prepared.

BSA: 1 mg/mL. 0.10 g bovine serum albumin (fraction V)/100 mL.

B) ASSAY

Standard curve	Tubes # (13 x 100 mm)						
Solution	1	2	3	4	5	6	7
BSA µL)	0	10	15	20	30	40	50
Water µL)	200	190	185	180	170	160	150
Solution F (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0

The solutions are vortexed and left 10 min at room temperature.

Solution D µL)	200	200	200	200	200	200	200

The solutions are vortexed and left at room temperature for 1 hour.

The absorbance of each solution is read at 750 nm using water as the blank.

UNKNOWN

Solution D.F.[a]	Tube # (13 x 100 mm)		
	1	2	3
Unknown (µL)	x	x	x
Water (µL)	y	y	y
	x + y = 200 µL		
Solution F (mL)	2.0	2.0	2.0

The solutions are vortexed and left 10 min at room temperature.

Solution D (µL)	200	200	200

The solutions are vortexed and left 1 hour at room temperature.

The absorbance of each solution is read at 750 nm using water as the blank.

The protein concentration is calculated using the standard curve and taking account of the dilution factor (D.F.).

a. D.F. (dilution factor). It has to be such so that the absorbance of the unknown at 750 nm is within the range of absorbance of the standards.

METHOD TO DETERMINE THE AMOUNTS OF MOLES OF AAMU OR 1X INCORPORATED PER MOLE OF BSA OR RSA.

This method gives an approximate estimate. It is a useful one because it allows one to determine whether the coupling proceeded as expected.

A) SOLUTIONS

- 10% sodium dodecyl sulfate (SDS)
- 1% SDS solution
- 0.5 or 1 mg/mL of AAMU-BSA (or AAMU-RSA) in a 1% SDS solution (1 mL).
- 0.5 or 1 mg/mL of BSA or RSA in a 1% SDS solution (1 mL).

B) PROCEDURE

- The absorbance of the AAMU conjugate solution is measured at 265 nm, with 1% SDS solution as the blank.
- The absorbance of the BSA (or RSA) solution is measured at 265 nm, with 1% SDS solution as the blank.
- The amount of moles of AAMU incorporated per mole of BSA (or RSA) is calculated with this formula:

$$y = \frac{A_{265}\ (\text{AAMU-BSA}) - A_{265}\ (\text{BSA})}{\varepsilon_{265}\ (\text{AAMU}) \times [\text{BSA}]}$$

Where:

y is the amount of moles of AAMU/mole of BSA (or RSA);

ε_{265} (AAMU) is the extinction coefficient of AAMU = 10^4 $M^{-1}cm^{-1}$; and

[BSA] = BSA (mg/mL)/68,000/mmole.

To calculate the amount of moles of 1X incorporated per mole of BSA or RSA, the same procedure is used but with this formula:

$$y = \frac{A_{252}\ (1X\text{-}BSA) - A_{252}\ (BSA)}{\varepsilon_{252}\ (1X) \times [BSA]}$$

Where:

y is the amount of moles of 1X/mole of BSA (or RSA);

ε_{252} (AAMU) is the extinction coefficient of 1X = 10^4 $M^{-1}cm^{-1}$; and

[BSA] = BSA (mg/mL)/68,000/mmole.

COUPLING OF HAPTENS TO HORSE RADISH PEROXIDASE

The AAMU derivative (VIII) and 1X derivative (IX) are conjugated to horse radish peroxidase (HRP) by the following procedure. To a 5 mL round bottom flask 31.2 mg of compound VIII (or 28.3 mg of compound IX) are added. Then 500 µL of dioxane, freshly dried over calcium chloride, are added. The suspension is stirred and cooled at 10°C using a water bath and crushed ice. Then 114 µL tributylamine and 31 µL of isobutyl chloroformate

(recently opened or purchased) are added. The suspension is stirred for 30 min at 10°C. While the suspension is stirring, a solution is prepared by dissolving 13 mg of horse radish peroxidase (HRP) in 2 mL of water. The solution is cooled at 4°C on crushed ice. After the 30 min stirring, 100 µL of a 1 N NaOH solution at 4°C is added to the HRP solution and the alkaline HRP solution is poured at once into the 5 mL flask. The suspension is stirred for 4 hours at 10-12°C. The free derivative is separated from the HRP conjugate by filtration through a Sephadex G-25™ column (1.6 x 30 cm) equilibrated and eluted with a 0.05 M sodium phosphate buffer, pH 7.5. The fractions of 1.0-1.2 mL are collected with a fraction collector. During the elution two bands are observed: the HRP conjugate band and a light yellow band behind the HRP conjugate band. The HRP conjugate elutes between fractions 11-16. The fractions containing the HRP conjugate are pooled in a 15 mL tissue culture tube with a screw cap. The HRP conjugate concentration is determined at 403 nm after diluting an aliquot (usually 50 µL+650 µL of buffer).

$$[\text{HRP-conjugate}](\text{mg/mL}) = A_{403} \times 0.4 \times \text{D.F.}$$

The ultraviolet (UV) absorption spectrum is recorded between 320 and 220 nm. The presence of peaks at 264 and 270 nm for AAMU-HRP and 1X-HRP conjugates,

respectively, are indicative that the couplings proceeded as expected.

After the above measurements, 5 µL of a 4 % thiomersal solution is added per mL of the AAMU-HRP or 1X-HRP conjugate solution. The conjugates are stored at 4°C.

ANTIBODY PRODUCTION

Four mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) are used for antibody production. The protocol employed in this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. Antibodies of the present invention may be monoclonal or polyclonal antibodies.

An isotonic saline solution (0.6 mL) containing 240 mg of BSA conjugated antigen is emulsified with 0.6 mL of a complete Freund's adjuvant. A 0.5 mL aliquot of the emulsion (100 mg of antigen) is injected per rabbit intramuscularly or subcutaneously. Rabbits are subsequently boosted at intervals of three weeks with 50 mg of antigen emulsified in incomplete Freund's adjuvant. Blood is collected by venipuncture of the ear 10-14 days after boosting. Antisera are stored at 4°C in the presence of 0.01% sodium azide.

DOUBLE IMMUNODIFFUSION IN AGAR PLATE

An 0.8% agar gel in PBS is prepared in a 60 x 15 mm petri dish. Rabbit serum albumin (100 µL of 1 mg mL^{-1}) conjugated to AAMU (or 1X) are added to the center well, and 100 µL of rabbit antiserum are added to the peripheral wells. The immunodiffusion is carried out in a humidified chamber at 37°C overnight and the gel is inspected visually.

ANTISERUM TITERS

The wells of a microtiter plate are coated with 10 µg mL^{-1} of rabbit serum albumin-AAMU (or 1X) conjugate in sodium carbonate buffer, pH 9.6) for 1 hour at 37°C (100 µL/per well). The wells are then washed three times with 100 µL TPBS (phosphate buffer saline containing 0.05% Tween ™ 20) and unoccupied sites are blocked by an incubation with 100 mL of TPBS containing 0.05% gelatin for 1 hour at 37°C. The wells are washed three times with 100 µL TPBS and 100 µL of antiserum diluted in TPBS are added. After 1 hour at 37°C, the wells are washed three times with TPBS, and 100 µL of goat anti-rabbit IgGs-alkaline phosphatase conjugate, diluted in PBS containing 1% BSA, are added. After 1 hour at 37°C, the wells are washed three times with TPBS and three times with water. To the wells are added 100 µL of a solution containing $MgCl_2$ (0.5 mM) and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min. at room temperature, the

absorbency is read at 405 nm with a microplate reader. The antibody titer is defined as the dilution required to change the absorbance by one unit (1 au).

ISOLATION OF RABBIT IgGS

The DE52-cellulose resin is washed three times with sodium phosphate buffer (500 mM, pH 7.50), the fines are removed and the resin is equilibrated with a sodium phosphate buffer (10 mM, pH 7.50). The resin is packed in a 50 x 1.6 cm column and eluted with 200-300 mL equilibrating buffer before use. To antiserum obtained from 50 mL of blood (30-32 mL) is added dropwise 25-27 mL of a 100% saturated ammonium sulfate solution with a Pasteur pipette. The suspension is left at room temperature for 3 h and centrifuged for 30 min. at 2560 g at 20°C. The pellet is dissolved with 15 mL sodium phosphate buffer (10 mM, pH 7.50) and dialyzed at room temperature with the buffer changed twice per day. The dialyzed solution is centrifuged at 2560 g for 10 min. at 20°C to remove precipitate formed during dialysis. The supernatant is applied to the ion-exchange column. Fractions of 7 mL are collected. After application, the column is eluted with the equilibrating buffer until the absorbance at 280 nm becomes less than 0.05 au. The column is then eluted with the equilibrating buffer containing 50 mM NaCl. Fractions having absorbencies greater than 0.2 at 280 nm are saved and stored at 4°C. Protein concentrations of the fractions are determined as described above.

COMPETITIVE ANTIGEN ELISA

Buffers and water without additives are filtered through millipore filters and kept for 1 week. BSA, antibodies, Tween™ 20 and horse radish peroxidase conjugates are added to these buffers and water just prior to use. Urine samples are usually collected 4 hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at -80°C. The urine samples are diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and are subsequently diluted with water to give concentrations of AAMU and 1X no higher than 3×10^{-6} M in the ELISA. All the pipettings are done with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well, 100 µL of a carbonate buffer (100 mM, pH 9.6) containing 2.5 µg mL^{-1} antibodies are added to each well. After 90 min. at room temperature, the wells are washed three times with 100 mL of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.05% Tween™ 20.

After the initial wash, unoccupied sites are blocked by incubation for 90 min. at room temperature with 100 µL TBP containing 3% BSA. The wells are washed four times with 100 µL TPB. The washing is followed by additions of 50 µL of 12 mg mL^{-1} AAMU-HRP or 1X-HRP conjugate in 2 x TPB containing 2% BSA, and 50 µL of either water, standard (13 standards; AAMU or 1X, 2×10^{-4} to 2×10^{-8} M) or sample in duplicate. The microplate is gently shaken with an orbital shaker at room temperature for 3-4 hours. The

wells are washed three times with 100 µL TPB containing 1% BSA and three times with water containing 0.05% Tween™ 20. To the washed plate is added 150 µL of a substrate buffer composed of citric acid (25 mM) and sodium phosphate dibasic buffer (50 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% o-phenylenediamine hydrochloride. After 20 min. at room temperature with shaking, the reaction is stopped with 50 µL of 2.5 M HCl. After shaking the plate 3 min., the absorbances are read with a microtiter plate reader at 490 nm.

RESULTS

Polyclonal antibodies against AAMU and 1X could be successfully raised in rabbits after their conjugation to bovine serum albumin. Each rabbit produced antibody titers of 30,000-100,000 as determined by ELISA. This was also indicated by strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin. On this basis, a) IgGs antibodies were isolated on a DE-52 cellulose column and b) a competitive antigen ELISA for NAT2 phenotyping using caffeine as probe substrate was developed according to the methods described in the above section entitled Materials and Methods.

Contrary to current methods used for phenotyping, the assay involves no extraction, is sensitive and rapid, and can be readily carried out on a routine basis by a

technician with a minimum of training in a clinical laboratory.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

A COMPETITIVE ANTIGEN ELISA FOR NAT2 PHENOTYPING USING CAFFEINE AS A PROBE SUBSTRATE

Buffers and water without additives were filtered through millipore filters and kept for 1 week. BSA, antibodies, Tween™ 20 and horse radish peroxidase conjugates were added to these buffers and water just prior to use. Urine samples were usually collected 4 hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at -80°C. They were diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and were subsequently diluted with water to give concentrations of AAMU and 1X no higher than 3×10^{-6} M in the ELISA. All the pipettings were done with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well, 100 µL of a carbonate buffer (100 mM, pH 9.6) containing 2.5 µg mL^{-1} antibodies was pipetted. After 90 min. at room temperature, the wells were washed three times with 100 µL of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.05% Tween™ 20.

After the initial wash, unoccupied sites were blocked by incubation for 90 min. at room temperature with 100 μL TBP containing 3% BSA. The wells were washed four times with 100 μL TPB. This was followed by additions of 50 μL of 12 mg mL^{-1} AAMU-HRP or 1X-HRP conjugate in 2 x TPB containing 2% BSA, and 50 μL of either water, standard (13 standards; AAMU or 1X, 2 x 10^{-4} to 2 x 10^{-8} M) or sample in duplicate. The microplate was gently shaken with an orbital shaker at room temperature for 3-4 hours. The wells were washed three times with 100 μL with TPB containing 1% BSA and three times with water containing 0.05% Tween™ 20. To the washed plate was added 150 μL of a substrate buffer composed of citric acid (25 mM) and sodium phosphate dibasic buffer (50 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% o-phenylenediamine hydrochloride. After 20 min. at room temperature with shaking, the reaction was stopped with 50 μL of 2.5 N HCl. After shaking the plate 3 min., the absorbances were read with a microtiter plate reader at 490 nm.

The competitive antigen ELISA curves of AAMU-Ab and 1X-Ab determinations obtained in duplicate are presented in Fig. 19. Each calibration curve represents the average of two calibration curves. The height of the bars measure the deviations of the absorbency values between the two calibration curves. Data points without bars indicate that deviations of the absorbency values are equal or less than the size of the symbols representing the data points. Under the experimental conditions of the ELISA: background was less than 0.10 au; the practical

limits of detection of AAMU and 1X were 2 x 10^{-7} M and 2 x 10^{-6} M, respectively, concentrations 500 and 50 times lower than those in urine samples from previous phenotyping studies (Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, 47:470-477); the intra-assay and interassay coefficients of variations of AAMU and 1X were 15-20% over the concentration range of 0.01-0.05 mM.

A variety of conditions for the ELISA were tested and a number of noteworthy observations were made: gelatin, which was used in the competitive antigen ELISA determination of caffeine in plasma (Fickling, S.A. et al. (1990) *J. Immunol. Meth.*, 129:159-164), could not be used in our ELISA owing to excessive background absorbency which varied between 0.5 and 1.0 au; in the absence of Tween™ 20, absorbency changes per 15 min. decreased by a factor of at least 3, and calibration curves were generally erratic; absorbency coefficients of variation of samples increased by a factor of 3 to 4 when the conjugates and haptens were added to the wells as a mixture instead individually.

The cross reactivities of AAMU-Ab and 1X-Ab were tested using a wide variety of caffeine metabolites and structural analogs (Table 8 below). AAMU-Ab appeared highly specific for binding AAMU, while 1X-Ab appeared relatively specific for binding 1X. However, a 11% cross reactivity was observed with 1-methyluric acid (1U), a major caffeine metabolite.

Table 8

Cross-reactivity of AAMU-Ab and 1X-Ab towards different caffeine metabolites and structural analogs

Compound	% Cross-Reaction AAMU-Ab	1X-Ab
Xanthine	0[a]	0
Hypoxanthine	0	0
1-Methyl Xanthine (1X)	0	100
3-Methyl Xanthine	0	0
7-Methyl Xanthine	0	0
8-Methyl Xanthine	0	0
1,3-Dimethyl Xanthine (Theophylline)	0	0.2
1,7-Dimethyl Xanthine (Paraxanthine)	0	0.5
3,7-Dimethyl Xanthine (Theobromine)	0	0
1,3,7-Trimethyl Xanthine (Caffeine)	0	0
Uric acid	0	0
1-Methyluric acid	0	11
1,7-Dimethyluric acid	0	0
Guanine	0	0
Uracil	0	0
5-Acetamino-6-amino-uracil	0.6	0
5-Acetamino-6-amino-1-methyluracil (AAMU)	100	0
5-Acetamino-6-amino-1,3-dimethyluracil	0	0

a. The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest compound concentration tested in the ELISA (5×10^{-3} M); concentrations of 5-acetamino-6-amino-1-methyluracil (AAMU) and 1-Methyl Xanthine (1X) required for 50% inhibition in the competitive antigen ELISA were 1.5×10^{-6} M and 10^{-5} M, respectively.

The relative high level of cross reactivity of 1U is, however, unlikely to interfere significantly in the determination of 1X and the assignment of NAT2 phenotypes, since the ratio of 1U:1X is no greater than 2.5:1 in 97% of the population (Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, 49:648-657). This is confirmed by

measurements of apparent concentrations of 1X when the ratio varied between 0-8.0 at the fixed 1X concentration of 3 x 10^{-6} M (Table 9 below). At 1U:1X ratios of 2.5 and 3.0, the apparent increases were 22% and 32%, respectively.

Table 9

The effect of the ratio 1U:1X on the determination of 1X concentration by ELISA at fixed 1X concentration of 3 x 10^{-6} M

1U:1X ratio	[1X] x 10^6 (M)
0.0	3.00
0.50	2.75
1.00	3.25
1.50	3.25
2.00	3.60
2.50	3.65
3.00	3.95
4.00	4.20
5.00	4.30
6.00	4.50
8.00	4.30

The following observations attested to the validity of the competitive antigen ELISA for NAT2 phenotyping.

1) The ELISA assigned the correct phenotype in 29 of 30 individuals that have been phenotyped by capillary electrophoresis (CE) (Lloyd, D. *et al.* (1992) *J. Chrom.*, 578:283-291).

2) In the CE method, the phenotype was determined using AFMU/1X peak height ratios rather than the AAMU/1X molar ratios used in the ELISA.

When the molar ratios determined by ELISA and the peak height ratios determined by CE were correlated by regression analysis, the calculated regression equation was $y = 0.48 + 0.87 x$, with a correlation coefficient (r) of 0.84, Taking account that these two ratios are not exactly equal and that Kalow and Tang (Kalow, W. *et al.* (1993) *Clin. Pharmacol. Ther.*, 53:503-514) have pointed out that using AFMU rather than AAMU can lead to misclassification of NAT2 phenotypes, there is a remarkable agreement between the two methods.

3) The ELISA was used in determining the NAT2 phenotype distribution within a group of 146 individuals. Fig. 20 illustrates a histogram of the NAT2 phenotypes of this group as determined by measuring the AAMU/1X ratio in urine samples by ELISA. Assuming an antimode of 1.80, the test population contained 60.4% slow acetylators and 39.6% fast acetylators. This is consistent with previously reported distributions (Kalow, W. *et al.* (1993) *Clin. Pharmacol. Ther.*, 53:503-514; Kilbane, A.J. *et al.* (1990) *Clin. Pharmacol. Ther.*, 47:470-477).

DETERMINATION OF 5-ACETAMINO-6-AMINO-1-METHYLURACYL (AAMU) AND 1-METHYL XANTHINE IN URINE SAMPLES WITH THE ELISA KIT

Table 10

Content of the ELISA kit and conditions of storage

Item	Unit	State	Amt	Storage conditions
Tween™ 20	1 vial	Liquid	250 µL/vial	4°C
H_2O_2	1 vial	Liquid	250 µL/vial	4°C
AAMU-HRP	1 vial	Liquid	250 µL/vial	4°C
1X-HRP	1 vial	Liquid	250 µL/vial	4°C
Buffer A	4 vials	Solid	0.8894 g/vial	4°C
Buffer B	6 vials	Solid	1.234 g/vial	4°C
Buffer C	6 vials	Solid	1.1170 g/vial	4°C
Buffer D	6 vials	Solid	0.8082 g/vial	4°C
Plate(AAMU-Ab)	2	Solid	-	4°C
Plate (1X-Ab)	2	Solid	-	4°C
Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
Standards (AAMU)	14 vials	Liquid	200 µL	-20°C
Standards(1X)	14 vials	Liquid	200 µL	-20°C
1N NaOH	1 bottle	Liquid	15 mL	20°C
1N HCl	1 bottle	Liquid	15 mL	20°C

CONVERSION OF AFMU TO AAMU

In order to determine the AAMU concentrations in urine samples by competitive antigen ELISA, a transformation of AFMU to AAMU is required. The contents of an ELISA kit for this assay are listed in Table 10.

- Thaw and warm up to room temperature the urine sample.
- Suspend the sample thoroughly with the vortex before pipeting.
- Add 100 µL of a urine sample to a 1.5 mL-microtube.
- Add 100 µL of a 1N NaOH solution.
- Leave at room temperature for 10 min.
- Neutralize with 100 µL 1N HCl solution.
- Add 700 µL of Buffer A (dissolve the powder of one vial A/50 mL).

DILUTIONS OF URINE SAMPLES FOR THE DETERMINATIONS OF [AAMU] AND [1X] BY ELISA

The dilutions of urine samples required for determinations of AAMU and 1X are a function of the sensitivity of the competitive antigen ELISA and AAMU and 1X concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X concentrations are about 3×10^{-6} M in the well of the microtiter plate. Generally, dilution factors of 100-400

(Table 11) and 50-100 have been used for AAMU and 1X, respectively.

Table 11
Dilution Factors for Identifying AAMU and 1X *Concentrations*

	Microtube #							
Dilution Factor	20x	40x	50x	80x	100x	150x	200x	400x
Solution	1	2	3	4	5	6	7	8
Urine sample(mL)[a] 10 x diluted	500	250	200	125	100	66.7	50	25
Buffer B (mL)	500	750	800	875	900	933.3	950	975

[a]. Vortex the microtubes containing the urine sample before pipeting.

Store the diluted urine samples at -20°C.

Buffer B: dissolve the content of one vial B/100 mL

DETERMINATION OF [AAMU] AND [1 X]IN DILUTED URINE SAMPLES BY ELISA

PRECAUTIONS

The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (Substrate buffer). Each sample is determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbance values of duplicates should be within less than 5%. Buffers C, D and E are freshly prepared. Buffer E-H_2O_2 is prepared just prior pipeting in the microtiter plate wells.

PREPARATION OF SAMPLES:

Prepare Table 12 with a computer and print it. This table shows the content of each well of a 96-well microtiter plate. Enter the name of the urine sample (or number) at the corresponding well positions in Table 12. Select the dilution factor (D.F.) of each urine sample and enter at the corresponding position in Table 12. Enter the dilution of each urine sample with buffer B at the corresponding position in Table 12: for example, for a D.F. of 100 (100 µL of 10x diluted urine sample + 900 µL buffer B), enter 100/900. See "Dilutions of urine samples..." procedure described above for the preparation of the different dilutions. Prepare the different dilutions of the urine samples in 1.5-mL microtubes. Prepare Table 13 with a computer and print it. Prepare the following 48 microtubes in the order indicated in Table 13.

Table 12

Positions of blanks, control and urine samples in a microtiter plate

Sample	Well #	D.F	Dil.	Sample	Well #	D.F	Dil.
Blank	1-2	-		Control	49-50		-
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72		-
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96		-

Table 13

Content of the different microtubes

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	AAMU or 1X	27	9	Dil. Urine
4	S2	AAMU or 1X	28	10	Dil. Urine
5	S3	AAMU or 1X	29	11	Dil. Urine
6	S4	AAMU or 1X	30	12	Dil. Urine
7	S5	AAMU or 1X	31	13	Dil. Urine
8	S6	AAMU or 1X	32	14	Dil. Urine
9	S7	AAMU or 1X	33	15	Dil. Urine
10	S8	AAMU or 1X	34	16	Dil. Urine
11	S9	AAMU or 1X	35	17	Dil. Urine
12	S10	AAMU or 1X	36	Control	Buffer B
13	S11	AAMU or 1X	37	18	Dil. Urine
14	S12	AAMU or 1X	38	19	Dil. Urine
15	S13	AAMU or 1X	39	20	Dil. Urine
16	S14	AAMU or 1X	40	21	Dil. Urine
17	S15	AAMU or 1X	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

SOLUTIONS:

Buffer A: Dissolve the content of one vial A/50 mL water.

Buffer B: Dissolve the content of one vial B/100 mL water.

Buffer C: Dissolve the content of one vial C/50 mL water. Add 25 mL of Tween™ 20.

Buffer D: Dissolve the content of one vial D /25 mL water. Add 25 mL of Tween™ 20.

0.05 % Tween™ 20: Add 25 µL of Tween™ 20 to a 100-mL erlenmeyer flask containing 50 mL of water.

2.5 N HCl: 41.75 mL of 12 N HCl/200 mL water. Store in a 250-mL glass bottle.

AAMU-HRP conjugate: Add 9 mL of Buffer C to a 15-mL glass test tube. Add 90 µL of AAMU-HRP stock solution.

1X-HRP conjugate: Add 9 mL of a 2 % BSA solution to a 15-mL glass test tube. Add 90 µL 1X-HRP stock solution.

Buffer E-H_2O_2: Dissolve the content of one vial E-substrate/50 ml water. Add 25 µL of a 30 % H_2O_2 solution (prepared just prior to adding to the microtiter plate wells).

Table 14

Standard solutions of AAMU and 1X
(diluted with buffer B)

AAMU		1 X	
Standard	[AAMU]	Standard	[1X]
1	1.12×10^{-4} M	1	2.00×10^{-4} M
2	6.00×10^{-5} M	2	1.12×10^{-4} M
3	3.56×10^{-5} M	3	6.00×10^{-5} M
4	2.00×10^{-5} M	4	3.56×10^{-5} M
5	6.00×10^{-6} M	5	2.00×10^{-5} M
6	3.56×10^{-6} M	6	1.12×10^{-5} M
7	2.00×10^{-6} M	7	6.00×10^{-6} M
8	1.12×10^{-6} M	8	3.56×10^{-6} M
9	6.00×10^{-7} M	9	2.00×10^{-6} M
10	3.56×10^{-7} M	10	1.12×10^{-6} M
11	2.00×10^{-7} M	11	6.00×10^{-7} M
12	1.12×10^{-7} M	12	3.56×10^{-7} M
13	6.00×10^{-8} M	13	2.00×10^{-7} M
14	3.56×10^{-8} M	14	1.12×10^{-7} M
15	2.00×10^{-8} M	15	6.00×10^{-8} M

CONDITIONS OF THE ELISA

Add 50 µL/well of AAMU-HRP (or 1X-HRP) conjugate solution, starting from the last row. Add 50 µL/well of diluted urine samples in duplicate, standards (see Table 14), blank with a micropipet (0-200 µL), starting from well # 96 (see Table 12). Cover the plate and mix gently by vortexing for several seconds. Leave the plate at room temperature for 3 h. Wash 3 times with 100 µL/well with buffer C, using a microtiter plate washer. Wash 3 times with 100 µL/well with the 0.05% Tween™ 20 solution. Add 150

µL/well of Buffer E-H_2O_2 (prepared just prior adding to the microtiter plate wells). Shake 20-30 min at room temperature with an orbital shaker. Add 50 µL/well of a 2.5 N HCl solution. Shake 3 min with the orbital shaker at room temperature. Read the absorbance of the wells with microtiter plate reader at 490 nm. Print the sheet of data and properly identify the data sheet.

CALCULATION OF THE [AAMU] AND [1X] IN URINE SAMPLES FROM THE DATA

Draw a Table 15 with a computer. Using the data sheet of the microtiter plate reader, enter the average absorbance values of blanks, controls (no free hapten present), standards and samples in Table 15. Draw the calibration curve on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma plot (or other plot software). Find the [AAMU] (or [1X]) in the microtiter well of the unknown from the calibration curve and enter the data in Table 16. Multiply the [AAMU] (or [1X]) of the unknown by the dilution factor and enter the result in the corresponding case of Table 16.

The compositions of the buffers used in the ELISA kit are shown in Table 17.

Table 15

Average absorbance values of samples in the microtiter plate

Sample	Well #	A_{490}	Sample	Well #	A_{490}
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

Table 16

AAMU (or 1X) concentrations in urine samples

Sample	D.F.	[AAMU]	[AAMU] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			

Table 17

Compositions of the different buffers

Buffer	pH	Composition	Concen. (mM)	[P] (mM)
A	7.50	0.15629 g/100 mL NaH_2PO_4	11.325	
		1.622 g/100 mL $Na_2HPO_4.7\ H_2O$	60.099	
		1.778 g/100 mL (total weight)		
				71.424
B	7.50	0.1210191 g /100 mL NaH_2PO_4	8.769	
		1.11309 g /100 mL of $Na_2HPO_4.7H_2O$	41.23	
		1.2341 g/100 mL (total weight)		
				49.999
C	7.50	1 g/100 mL of BSA	-	
		0.1210191 g /100 mL of NaH_2PO_4	8.769	
		1.11309 g /100 mL of $Na_2HPO_4.7H_2O$	41.23	
		2.2341 g/100 mL (total weight)		
				49.999
D	7.50	2 g/100 mL of BSA		
		0.1210191 g /100 mL of NaH_2PO_4	8.769	
		1.11309 g /100 mL of $Na_2HPO_4.7H_2O$	41.23	
		3.2341 g/100 mL (total weight)		
				49.999
E	5.00	0.52508 g/100 mL of citric acid	25	
		1.34848 g/100 mL of $Na_2HPO_4.7H_2O$	50	
		40 mg/100 mL of o-phenylenediamine hydrochloride		
		1.913567 g/100 mL (total weight)		-

The ELISA protocol outlined hereinabove, is adapted to provide a CYP3A4-specific ELISA, as well as other cytochrome P450 enzymes and N-acetylation enzymes of interest. In the case of CYP3A4, a CYP3A4-specific ELISA is provided for rapidly and accurately identifying CYP3A4 phenotypic determinants of an individual for use in

treating that individual with a dosage of an anticoagulant that is specific to at least their CYP3A4 phenotype.

Fig. 21 exemplifies a multi-determinant assay according to an embodiment of the present invention. A multi-determinant assay of the present invention may provide more than one 6 X 6 array, as illustrated in Fig. 22, in each well of a standard microplate. Preferably, each well will be provided with 4 6 x 6 arrays according to this aspect of the present invention.

The single or multi-determinant assay system of the present invention include(s) metabolite-specific binding agents for the detection of drug-specific metabolites in a biological sample. Such binding agents are preferably antibodies and the assay system is preferably an ELISA, as exemplified in the cases of NAT2 discussed herein above. A detection method according to an embodiment of the present invention is exemplified in Fig. 23. An assay system of the present invention is exemplified in Fig. 24 and provides means to detect metabolites specific to the metabolic pathway(s) used to metabolize anticoagulants.

The present invention provides a convenient and effective tool for use in both a clinical and laboratory environment. The present invention is particularly suited for use by a physician in a clinic, whereby phenotypic determinants for at least CYP3A4 can be quickly and easily obtained. According to an embodiment of the present invention, a ready-to-use kit is provided for fast and accurate determination of at least CYP3A4 determinants.

The assay system and kit preferably employ antibodies specific to a plurality of metabolites on a suitable substrate allowing for detection of the preferred metabolites in a biological sample of an individual after consumption of a corresponding probe substrate. In accordance with a preferred embodiment of the present invention, the kit of the present invention will provide means to determine metabolic determinants for at least CYP3A4. Alternatively, the kit of the present invention will provide means for determining phenotypic determinants of CYP3A4 and at least one of the following enzymes, CYP1A2, N-acetyltransferase-1 (NAT-1), N-acetyltransferase-2 (NAT-2), CYP2D6, CYP2A6, CYP2E1, CYP2C9 and CYP2C19. The assay system of the present invention may be provided in a plurality of forms including but not limited to an ELISA assay, a high-throughput ELISA assay or a dipstick based ELISA assay.

EXAMPLE II

DEVELOPMENT OF ANTIBODIES FOR DIFFERENTIALLY DETECTING R- & S-WARFARIN

Given the importance of warfarin as an anticoagulant, the detailed construction of an immunometric assay for its detection and the derivation of clinically significant information from its application requires further exemplification. Warfarin is used clinically in the form of a racemic (R,S) mixture. Studies have shown the S-enantiomer is metabolized via the CYP2C9 enzyme complex. The objective is to develop an immunometric technique to predict, for each patient, the correct warfarin dosage

which produces the desired anticoagulant effect. This is achieved by correlating therapeutic outcome to the temporal and fractional changes in the proportion of R- and S-warfarin and the major metabolite, S-7-OH-warfarin, measured in plasma after an initial warfarin dose.

GENERAL SYNTHESIS OF HAPTENS

The first step in development of an immunometric assay, consistent with the teachings of this invention, involves the design and synthesis of suitable analogs for application as haptens and, upon conjugation as immunogens. Towards this end, a series of novel (S)-, (R,S)-warfarin, and (R,S)-7-hydroxywarfarin derivatives were prepared. In order to preserve immunorecognition of the warfarin moiety at the principal metabolic site in the coumarin aryl ring, the linker was selectively introduced at the opposite 4' position. The key to this novel synthesis is the efficient elaboration of 4'-nitrowarfarin and its counterpart, 7-methoxy-4'-nitrowarfarin. These synthons are obtained in one step from 4-hydroxycoumarin and 4-hydroxy-7-methoxycoumarin, respectively, following the general sequence described diagrammatically in the Fig. 25.

The (S)-warfarin derivatives are prepared by chemical resolution of the racemic nitrowarfarins using quinidine salts.

SPECIFIC SYNTHESIS OF 3-[1-(4-AMINO-PHENYL)-3-OXO-BUTYL]-4-HYDROXY-CHROMEN-2-ONE (III) AND ITS AMIDATION WITH DECANEDIOIC (IV) ACID TO AFFORD 9-{4-[1-(4-HYDROXY-2-OXO-2H-CHROMEN-3-YL)-3-OXO-BUTYL]-PHENYLCARBAMOYL}-NONANOIC ACID (V)

A mixture of 4-hydroxycoumarin (500mg, 3mmol) and 4-nitrobenzalacetone (589 mg, 3 mmol) in water catalyzed by 5 mole per cent triethylamine is stirred at reflux for two days. Sodium hydroxide is added to obtain pH around 9. The aqueous layer is filtered and acidified (HCl) to pH 2. The resulting precipitate is collected by suction filtration to give 4'-nitrowarfarin (816 mg, 75 % yield).

4'-nitrowarfarin (816 mg, 2.31 mmol), 50 ml of dry THF, and 80 mg of Palladium (10 wt % on activated carbon) are placed in the steel reaction vessel of a high-pressure hydrogenation apparatus. The system is then closed, and hydrogen is admitted until the pressure, at 25°C, is about 20 PSI. The pressure in the reaction vessel is maintained at 20 PSI by the introduction of hydrogen from a tank for 24 hours. The catalyst is then separated from the reaction mixture by filtration through a Büchner funnel. Half the THF is removed by distillation, and methanesulfonic acid (222 mg, 2.31 mmol) is added dropwise. The resulting precipitate is collected by suction filtration to give 4'-aminowarfarin (III) as the methanesulfonate (675 mg, 70 % yield).

To a mixture of 4'-aminowarfarin methanesulfonate (675 mg, 1.61 mmol), sebacic acid (IV) as the monomethyl ester (348 mg, 1.61 mmol), and triethylamine (325 mg, 3.22 mmol), in 15 ml dichloromethane is added by portion at 0°C 1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide (309 mg, 1.61 mmol). After 24 hours, the organic phase is washed

with water, dried with sodium sulfate, and concentrated. The resulting crude syrup is purified by flash chromatography (hexane/chloroform/ethylacetate 1/1/2) to give warfarin monomethyl ester derivative (500 mg, 60 % yield).

This ester (500 mg, 0.95 mmol) is hydrolyzed using NaOH 1N solution for 24 hours. After acidification to pH 2, the resulting precipitate is collected by suction filtration to give warfarin hapten derivative (V) (400 mg, 85 % yield), with mass, proton and carbon NMR spectra in conformance with theory. The derivatized (S)-warfarin and (R)-warfarin enantiomers of (V) are subsequently elaborated from their corresponding chiral precursors of (III), which are, in turn, obtained by chemical resolution of the racemic nitrowarfarins using quinidine salts and serial recrystallization.

It follows by extension of this scheme that suitable nitrowarfarin precursors similar to (I) in Fig. 25 may be prepared with hydroxy groups in the coumarin aromatic ring preferably at the 7 position, which is the preferred cite of metabolism in the case of (S)-warfarin. Upon reduction to the amino moiety, it also follows that a series of homologous amides to the hapten structure (V) in Fig. 25 can be prepared in a similar manner by varying the linker moiety (IV) from 3 to 10 carbons in length and terminating in functional groups other than the carboxyl, such as hydroxyl, amino, and halogen bearing hydrocarbons suitable for conjugation onto the surface of carrier proteins.

CONJUGATION OF WARFARIN DERIVATIVES TO KLH CARRIER PROTEIN FOR IMMUNIZATION OF RABBITS FOR POLYCLONAL ANTIBODY PRODUCTION

Racemic warfarin, S-warfarin/C8 and racemic 7-hydroxywarfarin haptens prepared as described above were conjugated to Keyhole Limpet Hemocyanin (KLH) carrier protein in a 1 step reaction, using EDC as a catalyst. The carrier protein, KLH was dissolved in 0.9M NaCl, 0.1M MES buffer pH 5 at a concentration of 2, 5 and 10 mg/mL for the warfarin, S-warfarin and 7-hydroxywarfarin haptens, respectively. A hapten to KLH molar ratio of 2000:1 was used in each reaction. The haptens were dissolved in 100% DMSO at a concentration of 4 mg/mL for the warfarin and 8 mg/mL for S-warfarin and 7-hydroxywarfarin haptens. The final percentage DMSO in each reaction did not exceed 13%.

One skilled in the art will realize that other carrier proteins, including but not limited to, bovine serum albumin (BSA), can also be used in the present invention.

The catalyst, 1-Ethyl-3-(3-Dimethylaminopropyl) Carboiimide (EDC) was dissolved in 0.9M NaCl, 0.1M MES buffer pH 5 at a concentration of 10mg/mL. The EDC to hapten molar ratio used in the reactions were 5:1 for S-warfarin hapten and 8:1 for warfarin and 7-hydroxywarfarin haptens. The conjugation reaction was allowed to proceed for 2 hours, at room temperature with stirring. The conjugates were then purified by 3 cycles of dialysis in 0.9M NaCl, 83 mM Sodium Phosphate buffer at pH 7.2.

Conjugate concentrations were determined by the BCA protein assay and the percentage yield for each reaction was calculated. The incorporation ratios (IR) were

determined spectrophotometrically for each of the conjugates. The conjugates with the highest IR were selected for immunization at McGill University Animal Resources Center. Antigens, at a concentration of 200µg/mL in PBS buffer pH 7.2, were provided to the animal center for immunization. Following the immunization, boosts were injected every month, for a period of 12 months, and 10 mL of blood was collected 10 days following each boost.

Pooled blood samples were then elaborated and characterized in the usual manner and as previously described for application in immunometric assays.

One skilled in the art will realize that other animals, including other mammals and birds, can similarly be used to raise antibodies for differentially detecting R- and S-warfarin. One skilled in the art will also realize that immunogenic compositions for raising antibodies which bind to warfarin in an animal can contain the above-mentioned warfarin derivatives conjugated to a carrier moiety or protein in association with a pharmaceutically acceptable carrier. Antibodies can be raised by administering an immunogenic amount of the immunogenic composition to the animal. Such antibodies may then be isolated and may be polyclonal or monoclonal antibodies.

Hybridoma lines that produce the monoclonal antibodies are also envisioned by the present invention.

Intact or antigen binding fragments of these antibodies are envisioned by the present invention.. Such antigen binding fragments may be Fab fragments, $F(ab')_2$ fragments or Fv fragments.

Also envisioned are kits which utilize the antibodies of the present invention in assays for detecting

probe substrates and/or enzyme-specific metabolites thereof.

EXAMPLE III

USE OF METABOLIC PHENOTYPING IN DETERMINING INDIVIDUALIZED TREATMENT REGIMES WITH ANTICOAGULANT AGENTS

The exposure of an individual to a drug is described by the concept of area-under-the curve (commonly referred to as AUC). AUC is related to clearance by the following equation:

$$AUC = dose/clearance$$

Thus, if an individual's clearance is known, the dose can be individualized to achieve a desired AUC by the equation:

$$Dose = desired\ AUC \times clearance$$

An individual's rate of drug clearance is important as it determines the circulating drug concentrations. Both efficacy and toxicity are determined, in part, by the circulating concentrations of drug

Therefore, to individualize therapy a model is developed encompassing the numerous factors, which could possibly play a role in an individual's clearance value

for a particular medication(s) and hence predict a dose with maximal efficacy and minimal toxicity. As drug metabolism is the principal determinant of circulating drug concentrations, determining an individual's rate of drug metabolism is an important factor for the development of a successful model for the individualization of therapy. The model of the present invention will account for an individual's rate of CYP3A4 metabolism in determining a specific dose of an anticoagulant agent for that individual.

Other factors can alter drug clearance, such as body surface area, hepatic enzyme and protein levels (including serum alanine aminotransferases (ALT), albumin, alkaline phosphatases and serum α-1-acidicglycoprotein (AAG)), and drug transport proteins (including P-glycoprotein (pgp)).

Other individual specific characteristics may play a role in determining individual dose-limiting toxicity. According to another aspect of the present invention, other influencing factors may be accounted for, in addition to the rate of metabolism, in the model for the individualization of therapy with anticoagulant agents. For example, in the case of a specific anticoagulant such as warafin, other factors which may affect enzyme activity and hence toxicity include the activity level of warfarin's target enzyme, vitamin K epoxide reductase as well as other factors relating to this enzyme's activity such as polymorphisms in the enzyme microsomal epoxide

hydrolase (EPHX1), or overexpression of the protein calumenin.

Using multivariate analysis these individual factors will be examined for correlation to efficacy and toxicity. In accordance with one embodiment of the present invention, factors identified as having a significant correlation to either efficacy or toxicity will be included in the model along with drug metabolism.

The importance of drug metabolism in determining an individual's rate of drug clearance renders it as the most important factor in determining the efficacy and toxicity of many drugs. Some of the metabolic enzymes mentioned in the context of this invention have a clear bimodal distribution of metabolism, allowing the separation of the population into poor and extensive metabolizers. However, within each phenotypic group there is a wide variation in metabolic rates. It may be a naïve to regard all individuals with metabolic ratios greater than a predetermined cut off value as being equivalent. This attempt to classify the population in two or three phenotypic groups is even more difficult for enzymes without a bimodal distribution. The classification of individuals into this limited classification may not allow for the complete exploitation of an individual's pattern of metabolism. In some cases this simple classification is sufficient. For example, some individuals may have an enzyme specific deficiency, such as CYP2D6 and as a result are at risk for severe complications if high doses of a particular drug, such as Prozac™ are prescribed. However,

this simple classification would not allow for differential dosing of the extensive metabolizers as a function of the molar ratio calculated during determination of phenotype. If the simple classification of extensive CYP2D6 metabolizers was used, all individuals with a molar ratio of >0.3 (dextromethorphan as probe substrate) would receive the same dose. We are proposing the development of a dosing scale that would produce an increasing dose with increasing metabolic ratio, as exemplified in Fig. 25. If only the bimodal distribution is considered, only two possible doses can be prescribed. Accordingly, the individualization of therapy with anticoagulant agents is proposed in accordance with the present invention. As a result, the treatment with anticoagulant agents not based on phenotype will be replaced with individualization of treatment whereby the metabolism of each individual is assessed on an individual basis and a corresponding individual dosage is determined. In this manner, an anticoagulant agent is prescribed on an individual basis in dosages corresponding with an individual's phenotypic ability for metabolism.

In some cases multiple enzymes play key roles in determining the rate of drug metabolism. Therefore, the monitoring of only one metabolic enzyme in such cases may not provide complete information for individualizing therapy. The use of a multi-determinant assay examines multiple enzymes to provide additional metabolism-related information thereby providing a more accurate model for individualizing therapy is generated. For example, while the main metabolism of S-warfarin is performed by CYP2C9,

at least three other CYP450's (CYP2C8, CYP2C18 and CYP2C19) can play a minor role. Therfore, while most anticoagulant agents are metabolized primarily by CYP2C9 a multideterminant model may still play an important role for specific anticoagulants.

Individuals with extreme metabolic phenotypes are often at high risks for either toxicity or inefficacy of therapy. These ultraextensive or extremely poor metabolizers can often be identified by genotyping. For several metabolic enzymes genetic polymorphisms exist which result in an enzyme deficiency or the production enzyme with null activity. These individuals will not be affected by enzyme inducers or inhibitors and will consistently be extremely poor metabolizers. Identifying those individuals who carry these genetic polymorphisms allows physicians to avoid prescribing a drug metabolized by the enzyme in question. Conversely, several genetic polymorphisms have been identified that result in high levels of enzyme and/or increased enzyme activity. In addition, some individuals have been identified with multiple copies of the gene containing the polymorphism. As for the extremely poor metabolizers, these individuals may be excluded from certain treatment regimes due to increased risk of toxicity or lack of response.

Therefore, the use of genotyping to identify which individuals should be treated with a particular drug may be an excellent precursor to individualizing the individual's therapy based upon their specific phenotype. In doing so, an individual having a specific allelic

variation corresponding to an enzyme specific inefficiency in metabolism can be identified before undergoing preliminary phenotyping procedures and treatment with a probe substrate or substrate.

The knowledge of an individual's (multiple) phenotypic profile will allow physicians to:

1) determine if the individual has a phenotype that allows for the safe prescription of a drug;

2) determine the optimal drug dose in terms of drug efficiency and drug safety for an individual;

3) determine which drug of a plurality of drugs used for treating an individual's pathology or condition is the optimal drug in terms of drug efficiency and drug safety for that individual.

The knowledge of an individual's phenotypic profile for one or more enzymes will allow for the detection of drug(s) that could cause significant side effects or be inefficient in individuals with a specific phenotypic profile. In addition, the phenotypic profile will allow the development of an individualized dosing scheme with dose related to level of enzyme activities. The implementation of the multi-determinant phenotyping profile in treatment and dosing selection will lead to a marked decrease in side effects and increase in therapeutic efficiency.

WARFARIN

Although Warfarin is a widely used anticoagulatnt drug whose efficacy in the prevention of ischemic stroke in individuals with atrial fibrillation is well established, treatment with this oral anticoagulant is not without complications. There is a risk of serious hemorrhage which is reported to vary from 2.3-9.3% per year and the incidence of serious bleeding increases with the intensity of anticoagulation therapy, with the deviation in the prothrombin time ratio shown to be the strongest risk factor for bleeding complications. Moreover widespread inter-individual differences in the response to a given dose of warfarin are recognized and the effective daily doses of this racemic drug range from 1-60 mg/day.

Standardized induction regimens, with monitoring of International Normalized Ratio (INR) over the first four days, have only a 69% success rate in predicting the correct maintenance dose and, although complex nomograms and computer programs requiring protein C and protein S estimations have been shown to improve the prediction they are not suitable for routine use.

The INR is calculated by the following formuala:

$$INR = \left(\frac{\text{Individual's PT in seconds}}{\text{Mean Normal PT in Seconds}} \right)^{ISI}$$

INR = International Normalized Ratio
ISI = International Sensitivity Index

An individual's PT is determined using a test that measures the extrinsic clotting time of plasma. The normal range is 11 to 13.5 seconds ("normal" varies somewhat in different labs). For a person on full anticoagulant therapy the PT should be 2 to 3 times the laboratory "control" value. The INR value is a mathmatical "correction" (of the PT ratio) for differences in the sensitivity of thromboplastin reagents. This allows for the comparison of results between labs and standardizes reporting of the prothrombin time.

Researchers recognizing the need for a better predictor for warfarin-sensitivity have investigated inter-individual variations in warfarin metabolism. Warfarin is an enantiomeric mixture of R- and S-warfarin. However, the majority of the anticoagulant activity is present in the more potent S-warfarin enantiomer. S-warfarin is metabolized primarily by CYP2C9. Several researchers have investigated a correlation between CYP2C9 genotype and warfarin sensitivity. CYP2C9 has five known alleles, of which three are predominant, the wild type CYP2C9*1 allele and the mutant alleles CYP2C9*2 and CYP2C9*3 alleles which have been characterized as having decreased activity for S-warfarin metabolism. Several studies have demonstrated that the presence of the CYP2C9 mutant alleles CYP2C9*2 and CYP2C9*3 increases an individual's risk of bleeding complications during warfarin therapy as well as resulted in a lower maintenance dose required (see Table 18) (Daly and King 2003, Pharmacogenetics 13:247-252).

Table 18

Effects of the genetic polymorphism of cytochrome P450 CYP2C9 on the *in vivo* pharmacokinetics and pharmacodynamics of warfarin

No. of Individuals	Ethnic Background	Pharmacokinetic or Pharmacodynamic Changes
94	British	MD; 3.8 (*1/*2) vs. 4.7 (*1/*1) mg/day
1	Not Specified	7.8 fold ↑ in plasma (S)-/(R)- ratio in individuals with *3/*3 MD; 0.5 (*3/*3) vs. 4-8 (*1/*1) mg/day
86	Japanese	63% ↓ in $CL_{po,u}$ for (S)-warfarin in 3 individuals with *1/*3
47	Japanese	90% ↓ in $CL_{po,u}$ for (S)-warfarin in an individual with *3/*3 MD; 0.4 (*3/*3) vs. 1.75 (*1/*3) vs. 3.0 (*1/*1) mg/day
88	British	81% of low dose (1.5 mg/day) group had at least 1 of *3 or *2 allele vs. only 38% of control group; ↑ *risks of major bleeding in the low dose group* (↑ probability of encountering difficulties in titrating warfarin dose at induction)
52	British	MD; 2.5 (*1/*3) vs. 3.5 (*1/*2, *2/*2) vs. 4.25 (*1/*1) mg/day
38	Caucasian and African-American	MD; 0.307 (*1/*3, *1/*2, *2/*2, CYP2A6*1/*2) vs. 0.397 (*1/*1) mg/kg/wk
561	British	MD; 3.97 (*1/*3) vs. 4.31 (*1/*2) vs. 3.04 (*2/*2) vs. 4.09 (*2/*3) vs. 5.01 (*1/*1) mg/day
180	Caucasian	MD; 1.8 (*2/*3) vs. 3.8 (*1/*3) vs. 5.2 (*1/*2, *2/*2) vs. 6.7 (*1/*1) mg/day ↑ risk of bleeding in individuals with at least 1 of *3 or *2 allele (odds ratio 2.57; (5% CI 1.16-5.73)

CI=Confidence Interval; $CL_{po,u}$ =unbound oral clearance; MD=daily maintenance dose

The clinical implications of the CYP2C9*4 and CYP2C9*5 alleles remains to be studied. The genotyping studies have suggested that an individual's CYP2C9 genotype be determined prior to warfarin dosing and that the starting warfarin dose be dictated according to the classification of slow and fast metabolizers.

In the current invention we are proposing that warfarin be dosed according to an individual's CYP2C9 phenotype. The use of genotype is complicated by the necessity of studying a range of alleles (for example, the impact of CYP2C9*2 and CYP2C9*3 have been investigated, however, the recently discovered CYP2C9*4 and CYP2C9*5 alleles have yet to be investigated). Phenotyping allows for the summation of all genotypes into one measurement and regardless of the individual's genotype a phenotypic measurement is a true measurement of an individual's CYP2C9 functional levels. Additionally phenotyping summates the impact of enzyme induction or inhibition (example, CYP2C9 is inhibited by the NSAIDS diclofenac or ibuprofen), while genotyping fails to account for any impact from polypharmacy. This has the potential of having a high impact on warfarin sensitivity as bleeding complications are more prevalent in the elderly, who are also more prevalent to being treated with multiple medications.

EXAMPLE IV

PREDICTIVE VALUE OF THE CYP2C9 PHENOTYPE ON THE DETERMINATION OF A STABLE, EFFECTIVE AND SAFE DOSE OF WARFARIN

DEVELOPMENT OF A THERAPEUTIC NOMOGRAM

We are proposing the dosing of warfarin according to a predetermined CYP2C9 phenotype using a dosing nomogram. We predict that this scheme for dosing warfarin will result in a decreased time to establishing a maintenance dose, a decrease in the trial and error involved in determining the daily maintenance dose and a decrease in the incidence of bleeding complications during the establishment of the daily maintenance dose.

Warfarin is an oral anticoagulant in the coumarin family used in the prophylactic and curative treatment of pulmonary embolism and venous and arterial thrombosis. Warfarin use requires careful individual monitoring and frequent dosage adjustments. In fact, the daily dose required to maintain an acceptable level of efficacy while avoiding a high risk of side effects varies from one individual to another (0.5-60 mg). Furthermore, the initial dosage often remains empirical.

Warfarin is used clinically in the form of a racemic mixture consisting of two isomers: S-warfarin (50%) and R-warfarin (50%). The pharmacologic activity is found mainly at the level of the S-warfarin isomer. This isomer is 2 to 5 times more powerful than the R isomer. However, R-warfarin predominates at steady state with a

plasma concentration that is approximately double that of S-warfarin.

Numerous factors, including age, sex, genetic variants, illnesses and drug interactions may affect response to warfarin. These factors may lead to changes both in the pharmacokinetics of the drug (absorption, metabolic clearance) and in its pharmacodynamics (different responses to similar concentrations). Thus, warfarin dosing must be adapted to the needs of each individual based on the individual's level of sensitivity to this drug.

Determination of the International Normalized Ratio (INR) is the best way to measure an individual's sensitivity to warfarin. The risk of bleeding increases exponentially when INR exceeds 5.0. However, INR values must be maintained above a critical minimum level to prevent thromboembolism. Thus, the target level for INR is generally between 2.0 and 3.5 for the majority of individuals, depending on therapeutic indications. For deep vein thrombosis, atrial fibrillation and myocardial infarction, the target INR is between 2.0-3.0, while the INR for individuals with mechanical valves is between 2.5-3.5.

A number of algorithms suggesting initiation doses for oral anticoagulant therapy have been proposed in the past, but their performance has often been disappointing. The strategies used include the development of graphic approaches or the use of computers, standardization based on specific physiological parameters of the individual

such as age, standardized initial dosage, systematic follow-up by different professionals, or self-adjustment by the individuals themselves. Initiation of warfarin therapy with the tools available is therefore often empirical and the time needed to achieve a stable anticoagulant effect is frequently very long (6 to 8 weeks). In this situation, individuals have to undergo numerous blood samplings that may negatively impact their quality of life. Furthermore, particularly sensitive individuals may have an excessive reaction to average initial doses, and thus be exposed to an increased risk of bleeding.

The cytochrome P450 superfamily is responsible for the metabolism of a wide variety of drugs, including warfarin. Some 20 subfamilies have been identified in humans, but only four of them are primarily involved in drug metabolism: CYP1, CYP2, CYP3 and CYP4. Metabolism studies have clearly indicated that the two warfarin isomers are metabolized by different isoenzymes. CYP2C9 is the enzyme principally responsible for the metabolism of S-warfarin, while CYP1A2, CYP3A4 and CYP2C19 are responsible for the metabolism of R-warfarin. CYP2C9 represents one of the major isoforms in the CYP2C subfamily which accounts for approximately 20% of human liver CYP450 activity. This isoform has several substrates other than S-warfarin such as losartan, phenytoin, nonsteroidal anti-inflammatories, celecoxib and sulfonylureas. The interactions between S-warfarin (active isomer) and the other CYP2C9 substrates may

therefore represent an important factor in the variations in response to warfarin.

One of the important causes of interindividual variations in the effects of a drug is genetic variation in the activity of the enzymes responsible for its metabolism. It is now recognized that genetic polymorphism of enzymes may generate separate subgroups in the population, who differ in their ability to transform certain drugs. Mutations in the gene coding for these enzymes may cause a reduction, increase, or complete inhibition of the pharmacologic activity of a drug metabolized by this enzyme. CYP2C9 is the subject of genetic polymorphism. In fact, six allele variants have been identified thus far: the wild-type allele, CYP2C9*1, and CYP2C9*2-6 variants. Two of these allele variants are associated with a loss of activity (CYP2C9*3 and CYP2C9*6) while CYP2C9*2, CYP2C9*4 and CYP2C9*5 are associated with weaker enzyme activities. Transmission is recessive and autosomal, and individuals can therefore be homozygous or heterozygous for one or more of these variants. It has now been accepted that the differences observed in warfarin metabolism can be explained by the differences in the activities associated with the various genotypes. Thus, variations in *in vitro* metabolism and in the kinetic parameters of warfarin (plasma concentration, metabolism speed and intrinsic clearance) have been observed depending on the allelic variant. Last, more and more clinical reports and clinical studies indicate the need for lower doses of warfarin in individuals with allelic variants of CYP2C9. For instance, a recently summarized

clinical study (Redman (2001) Pharmacotherapy 21:235-242) involved 94 subjects being followed by two different anticoagulant therapy clinics, whose INR had to be maintained between 2.0-4.0 for three consecutive days. The results of the study are summarized in Table 19 below.

Table 19

Summary of Clinical Study Following Individuals at Different Anticoagulant Therapy Clinics

Genotype	Incidence of alleles	Associated dose of warfarin
Homozygoous for CYP2C9*1	62%	31.6 mg/week or 4.50 mg/day
Heterozygous for CYP2C9*2	38%	26.3 mg/week or 3.75 mg/day
Homozygous for CYP2C9*2 and CYP2C9*3	Not detected	

A high incidence (90%) of subjects requiring a low dose of warfarin (5-15 mg/week) proved to be heterozygous for CYP2C9*2. Inversely, 83% of the subjects who required a high dose of warfarin (> 55 mg/week) proved to be homozygous for CYP2C9*1. In short, it appears that subjects with at least one variant allele other than the wild type (CYP2C9*1) have reduced S-warfarin metabolism, have higher concentrations of this isomer and generally require lower doses of warfarin. The impact on the health of these subjects is important, since when standard doses are used, the risk of bleeding increases, hospitalization may be prolonged and the quality of life of these individuals is reduced. Furthermore, the treatment of these complications may increase health care costs.

The literature currently focuses only on individual genotyping based on INR values and plasma

concentrations of warfarin and then, only in small samples. This project therefore intends to assess the relationship between the CYP2C9 phenotype (as determined by the urinary metabolic ratio of losartan) and the dose of warfarin required to achieve the desired level of anticoagulation. The phenotype determination represents a measurement sensitive not only to genotype but also to all of the factors that may alter the activity of the enzymes involved in drug metabolism (drug interactions, age, pathology, etc.). Furthermore, the proposed study will be conducted on a larger sample of subjects in order to increase the chances of encountering individuals who are very sensitive or resistant to warfarin.

In short, a better understanding of the clinical impact of factors that may cause interindividual variations in their response to warfarin will certainly make it possible to obtain a more rapid and stable anticoagulant affect. This may contribute to minimizing the risks associated with initiation of therapy and thus provide individuals with safer anticoagulant therapy. The following project was intended to develop a nomogram that can define a customized strategy for warfarin initiation.

PRINCIPAL OBJECTIVE

The principal objective of the study was to show that the CYP2C9 phenotype makes it possible to predict the dose of warfarin that produces the desired anticoagulant effect once the individual has been stabilized. One hundred and fourty subjects were recruited to develop a

treatment nomogram based on the relationship established between warfarin dosage, INR at 14 days and CYP2C9 phenotype.

DEFINITIONS OF VARIABLES AND COVARIABLES

VARIABLES

DETERMINATION OF CYP2C9 PHENOTYPE

A phenotype of each individual was determined according to the urinary metabolic ratio of losartan (CYP2C9 surrogate probe substrate) as well as the plasma ratio of R- & S- warfarin (drug as own probe substrate). A single dose of 25 mg losartan was administered before initiating warfarin therapy and urine was collected for a period of 6 hours. After the intial loading dose of warfarin (5mg) blood was collected at 3, 14 and 24 hour time points.

DETERMINATION OF GENOTYPE

CYP2C9 genotyping based on DNA was performed for the following genetic variants: CYP2C9*1, CYP2C9*2, CYP2C9*3, CYP2C9*4, CYP2C9*5 and CYP2C9*6. The DNA was extracted and isolated from leucocytes isolated from a blood sample. The six variants were amplified by PCR (polymerase chain reaction) and digested for detection.

INR

The INR test was performed from an intravenous puncture according to standard hematology lab procedures at the Institut de cardiologie de l'Hôpital Laval and the

Montréal Heart Institute. The results of this test were recorded throughout the titration period in the subject's research file, and a mandatory analysis was performed at day 14 after initiation of warfarin therapy.

COVARIABLES

- Age may affect the maintenance dose of warfarin. This covariable was therefore considered a constant in the analysis.
- The gender of the subjects may have a considerable effect on predicting warfarin dosage when therapy is initiated. It therefore was considered in the analysis.
- Genetic variants vary by ethnic group. Some studies have shown that the incidence of genetic variants differs from one ethnic group to another. Ethnic group was considered a covariable and was determined by directly questioning the subject. Subjects were categorized as follows:
    - Caucasian
    - Black (African-American)
    - Asian
    - Other

  In the event of mixed ethnic origin, the ethnic origin of the parents (first generation) were documented.
- Alcohol consumption can affect drug metabolism because ethanol is metabolized by CYP2E1 and may induce CYP2C9.

  Alcohol consumption was categorized as follows:

- No consumption
- Occasional consumption
- 1 to 2 drinks per day
- >2 drinks per day

Alcohol consumption was determined by direct questioning.

One drink corresponds to 250 mL wine, 375 mL beer, or 30mL spirits.

- Modification of smoking habits may affect the INR of individuals taking anticoagulants. Tobacco use was based on the number of cigarettes smoked per day by direct questioning of the subjects. Tobacco use was categorized as follows:
  - Nonsmoker
  - 1-10 cigarettes per day
  - >10 cigarettes per day
- Indication for anticoagulation:

  The indication for anticoagulation was identified from the follow-up form from the anticoagulant therapy clinic or from the anticoagulant prescription filled out by the physician.
  - Deep vein thrombosis
  - Atrial fibrillation
  - Mechanical heart valve
  - Other
- DIET

Anticoagulants in the coumarin family are vitamin K antagonists that produce their anticoagulant effect by interfering with the interconversion cycle of vitamin K and its epoxides. Green leafy vegetables contain a great deal of vitamin K and any variation in the weekly consumption of these vegetables may significantly affect INR.

The consumption of green leafy vegetables was determined by direct questioning of the subjects. Consumption was categorized as follows:

- Never
- Occasionally (1-3 times / week)
- Regularly (4-7 times / week)

- Medications affecting warfarin metabolism:

The analysis systematically took into account the use of agents recognized for their significant effect on CYP2C9 activity.

- Nonsteroidal anti-inflammatories
- Sulfonylureas
- Celecoxib
- SRIs (fluoxetine, fluvoxamine, paroxetine)
- Amiodarone
- Propafenone
- Fluconazole
- TMP/SMX
- Metronidazole

- Macrolide antibiotics (erythromycin, azithromycin, clarithromycin)
- Phenytoin
- Rifampicin
- Carbamazepine

All changes in pharmacological profile were documented while subjects were being monitored.

- Generic formulation:

The use of the original warfarin product (Coumadin™) or a generic formulation was documented at the first meeting.

METHODOLOGY

Research specifications: Prospective cohort study.

Target population: Individuals hospitalized at Hôpital Laval or the Montréal Heart Institute in whom warfarin therapy is initiated.

INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria:

- Individuals in whom anticoagulant therapy is initiated in the hospitals participating in the study.
- Estimated duration of treatment: at least 14 days
- Age (18 years and over)
- Individuals willing to take warfarin in the evening between 5-8 p.m.

• Exclusion criteria:

- Warfarin allergy
- Pregnant women
- Individuals whose INR is not measured by the hematology laboratory of *Hôpital Laval* or the Montréal Heart Institute.
- Individuals who do not speak French or English
- Individuals involved in other research projects
- Individuals whose warfarin therapy has been temporarily discontinued due to surgery or an invasive procedure.

EXPERIMENTAL SECTION

GENOTYPING

The CYP2C9 genotyping can be performed but, not limited to, using the Polymerase-Chain-Reaction (PCR) – Restriction Fragment Length Polymorphism (RFLP) technique from an extract of DNA. Other methods known in the art, such as but, not limited to, single nucleotide polymorphism (SNP) or cleaved amplified polymorphic DNA (CAP) analysis, can also be used determine an individual's CYP2C9 genotype. The DNA can be isolated from the leucocytes by extraction using standard methods.

The alleles of interest were amplified by PCR and the products (DNA fragments) of the latter were digested with *Ava*II and *Kpn*I enzymes to detect CYP2C9*2 and CYP2C9*3 respectively. The products (DNA fragments) were separated on a 10% acrylamide gel (TBE 1X) (Scordo MG, Aklillu E, Yasar U, *et al.* (2001) Br J Clin Pharmacol 52:447; Garcia-Martin E, Martinez C, Ladero JM, *et al.* (2001) Eur J Clin Pharmacol 57:47. The techniques were also adapted to detect CYP2C9*4, CYP2C9*5 and CYP2C9*6 alleles (Kidd RS, Curry TB, Gallagher S, *et al.* (2001) Pharmacogenetics 11:803; Dickmann LJ, Rettie AE, Kneller MB, *et al.* (2001) Mol Pharmacol 60:382).

DETERMINATION OF THE URINARY METABOLIC RATIO OF LOSARTAN

Urine concentrations of losartan and its metabolite were determined using the HPLC method. In short, the compounds were separated by inverse phase gas chromatography on a CN column with detection in UV mode at a length of 245 nm. The quantification limits are less than 10 ng/mL (Soldner A, Spahn-Langguth H, Mutschler E, (1998) J Pharm Biomed 16:863).

DETERMINATION OF THE PLASMA METABOLIC RATIO of WARFARIN

Plasma concentrations of R- & S-warfarin were determined by HPLC. In brief, 700 µl 1N sulphuric acid was added to 1 ml of sample, this mixture was briefly vortexed & 4 ml of ethyl ester added subsequently. The solution was centrifuged at 3000 rpm for 5 min, flash frozen and the upper organic layer collected and evaporated at 40°C with nitrogen gas. The pellet was reconstituted in 200 µl of acetonitrile with sonication.

The sample was then analyzed by HPLC using an autosampler and under the following conditions:

- Column: CYCLOBOND I 2000, 250mm X 4.6 mm
- Wavelength of DAD: 320 nm
- Injection Volume: 50 µl
- Run Time: 17 min
- Excitation / Emmision used: 320 nm / 415 nm
- Flow Rate: 2.0 ml/min

- Autosampler temperature: 4°C
- Mobile Phase:

1000:3:2.5, Acetonitrile: Glacial Acetic Acid:Triethylamine

- Syringe Rinse: 50:50, Acetonitril: water (Grade 1)

Standard curves of R-, S- warfarin were calculated from the peak area of each standard and its internal standard using the formula $y=mx + b$, using a $1/x^2$ weighting or equivalent. Where,

y= response rate

x= concentration

b= response (predicted) at zero concentration (B_0)

m= slope of the plot ($\delta Y/\Delta X$)

Concentrations of R- & S- warfarin in the samples was determined by back calculating from the appropriate standard curve, and recovery-correction using the internal standard result.

MEASUREMENT OF ANTICOAGULANT ACTIVITY

INR was measured at the first meeting and at day 14. In addition, tests were performed as required, as dictated by good clinical practice. The INR assay was performed by the hematology laboratory according to standard clinical procedures from the venous blood samples.

DATA ANALYSIS

Multiple regression analyses were performed by determining as criterion variables the warfarin dose at day 14. The independent variables in these models will be the CYP2C9 phenotype as determined by the urinary metabolic ratio of losartan and INR or the CYP2C9 phenotype as determined by the plasm metabolic ratio of warfarin and INR. Secondly, additional covariables were evaluated such as CYP2C9 genotype, gender, age and administration of other drugs. The threshold for statistical significance will be established at $P<0.05$.

DETERMINATION OF WARFARIN METABOLISM IN HUMAN SUBJECTS AND CORRELATION OF PARTIAL CLEARANCES SO DERIVED TO OUTCOME MEASURES OF ANTI-COAGULATION STATUS

Experts in the field of hematology have come to understand that, as the (S)-warfarin component of a therapeutic racemic warfarin dose is preferentially metabolized, the molar ratio (R/S), and therefore the molar fraction (R/(R+S)) of warfarin in serum increases as a function of time after a bolus dose of the racemic drug. Rapid metabolizers will show a greater R fraction than slow metabolizers, and therefore a slower clearance. It should follow that rapid metabolizers, having cleared the therapeutic drug faster, would be less likely to sustain the desired anti-coagulation response than slow metabolizers, who in turn would be at greater risk for excessive anticoagulation. In either case, inappropriate maintenance of a desired coagulation state is generally recognized as detrimental and the cause of both mortality

and significant morbidity associated with the use of a narrow therapeutic index drug.

Noting that variation in the concentrations of warfarin enantiomers can range between 6 and 2000 ng/ml within three hours of a single bolus dose as low as 5 mg, an appropriate assay system to recognize R, S and RS warfarin was developed and validated to encompass that range of dilutions. Those practiced in the art will recognize that such a range is commonly addressed by standard ELISA assays for numerous analytes in clinical chemistry practice. Similarly, HPLC and tandem MS methodologies will also serve as an appropriate chemometric technology for analytes in this range of concentration.

Accordingly, after validating measurements in blanks and sham experiments, a population of 73 individuals undergoing treatment with warfarin was examined. The individuals were being initiated on anticoagulation therapy in response to atrial fibrillation and similarly appropriate cardiac indications. After an initial loading dose of 5 mg, each individual was then treated in accordance with the usual dosing nomograms and monitored solely by periodic INR measurements. In parallel, the concentrations of warfarin enantiomers in blood were determined at 3, 14 and 24 hours post the first dose. The clearance index as measured disposal rates (CLM_R) for each individual was then determined as Initial Dose divided by chiral warfarin concentration at each of the time points, to give three sets of characteristic values-- CLM_RW_3, CLM_RW_14 and CLM_RW24 . After two weeks on a standard therapy regimen designed to achieve a maintenance INR of 2-3, the actual INR values for the population were clustered into three outcome sets: Set 1 for individuals with INR <2, Set 2 for individuals with INR>2 and INR<3, and Set 3 for

individuals with INR>3. It follows from this clustering that individuals in Set 1 should be considered to have been underdosed, in part attributable to their fast warfarin metabolism, while individuals in Set 3 should be considered to have been overdosed in part because of their fast metabolism. Fig. 27 shows the outcome of the analytical experiment. The individuals in Set 1, who were underdosed by the normal course of therapy, all showed significantly higher clearance index values. Individuals that were overdosed showed a significantly lower clearance index. Results using losartan phenotyping showed similar results.

Thus, in terms of proportional dosing, the information obtained from the metabolism of a single test would have proven useful in arriving at an appropriate dose. If the fast metabolizers had been given approximately double the otherwise standard dosing regimen, their clearance index would have approximated that which is typical of the subset of individuals that were, in fact, appropriately dosed. Conversely, if the individuals who were clinically underdosed by the traditional dosing scheme had been given twice the indicated dose, then they too would have attained a clearance index in concert with the norm set for adequately dosed individual. Thus, by first monitoring the metabolic capacity of each individual to dispose of warfarin, at either 3, 14, or 24 hours post a first, trial dose, then the total therapeutic demand can be met so as to achieve a more uniform outcome in the desired and safe level of anticoagulation.

The present invention provides for an individualization model based upon at least an individual's specific CYP2C9 phenotype for use in the individualization of therapy with anticoagulant agents.

This proactive procedure will identify starting doses much more accurately than the standard methods, and will result in much less post-administration "fine-tuning" of the dose.

In accordance with one embodiment of the present invention, prior to undergoing warfarin therapy individuals are administered a predetermined dose of a CYP2C9 specific probe substrate. A biological sample is collected (e.g. urine) after the probe substrate is consumed. The concentrations of the probe substrate and metabolite(s) are determined and a molar ratio calculated. This molar ratio is specific to the individual's level of CYP2C9 activity.

To determine the rate of CYP2C9 activity, losartan may be used as a probe substrate and the molar ratio of hydroxylosartan, the losartan metabolite, and losartan (hydroxylosartan/losartan) calculated. Alternatively, warafarin may be used as its own probe substrate and the chiral ratio of R- & S-warfarin calculated. An individual's ratio is indicative of their CYP2C9 enzyme activity, with a lower ratio indicating poorer metabolism and a higher ratio indicating more extensive metabolism. The activity of CYP2C9 metabolism is distributed unimodally and hence no antimode is present. The levels of CYP2C9 activity as determined by direct phenotyping will be incorporated into an individualization of therapy model of the present invention to determine a treatment dosage of an anticoagulant that correlates with an individual's ability to metabolize that anticoagulant. An

ELISA system as exemplified above may be employed to detect phenotypic determinants of at least CYP2C9 for determining an individual's CYP2C9 metabolic activity. The present invention provides for an individualization model based upon at least an individual's specific CYP2C9 phenotype for use in the individualization of therapy with anticoagulants. The individualization model of the present invention may further include other enzyme-specific determinants as well as other factors, which have a significant contribution to the clearance of anticoagulant agents in the body or a significant contribution to toxicity (e.g. pretreatment renal function).

In accordance with an embodiment of the present invention, an assay system is provided that can be used in a clinical environment, whereby phenotypic determinants can be quantified from a urine sample and applied to an individualization model to determine a dosage of an anticoagulant agent for treating an individual which at least corresponds to the individual's ability to metabolize CYP2C9. As a result, physicians will be provided with a tool for the individualization of therapy providing an alternative to the arbitrary selection of medications based on prognosis and categorical dosing.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures

from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.